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PROCEEDINGS OF THE THIRTEENTH CONFERENCE ON ENVIRONMENTAL TOXICOLOGY 16, 17, and 18 NOVEMBER 1982

UNIVERSITY OF CALIFORNIA, IRVINE
OVERLOOK BRANCH, P.O. BOX 31009
DAYTON, OHIO 45431

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AIR FORCE AEROSPACE MEDICAL RESEARCH LABORATORY
AEROSPACE MEDICAL DIVISION
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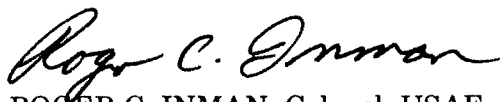
The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER



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Papers were presented covering current methods in immunotoxicology, immune mediated effects of specific chemicals, comparative effects of inhaled toxicants in different species, approaches to the mechanisms of toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin and related chemicals, and toxicity of hydrocarbons and hydrocarbon metabolites.

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PREFACE

The Thirteenth Conference on Environmental Toxicology was held in Dayton, Ohio on 16, 17, and 18 November 1982. Sponsor was the University of California, Irvine under the terms of Contract No. F33615-80-C-0512, Work Unit 63020115 with the Aerospace Medical Research Laboratory, Aerospace Medical Division, Air Force Systems Command, Wright-Patterson Air Force Base, Ohio, partially funded by the U. S. Naval Medical Research Institute, Toxicology Detachment, Wright-Patterson Air Force Base, Ohio under MIPR No. N6433482MP00009.

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WELCOMING REMARKS

Maj. Gen. John W. Ord
USAF, MC

Good morning, ladies and gentlemen. I am pleased to have this opportunity to welcome you on behalf of the Air Force's Aerospace Medical Division to the 13th Conference on Environmental Toxicology. The sponsor for this year's meeting is the University of California at Irvine, under terms of the contract with the Air Force Aerospace Medical Research Laboratory.

Although the conference is described as the 13th Annual Conference in Environmental Toxicology, this is really the 18th meeting. The first five conferences did not carry the "Environmental" label since they concentrated on the toxicology problems associated with the early man-in-space programs. Following cancellation of the manned orbiting laboratory program in 1969, and coincident with growing national interest in environmental quality and health, our Air Force program was reoriented toward the severely challenging environmental toxicology issues. The unique exposure facilities which were established in the Toxic Hazards Division to support the space effort then became a truly outstanding resource in the area of inhalation toxicology. It is interesting to note that the Air Force has established a new man-in-space thrust this year with the establishment of Space Command, and much of the toxicology data base established earlier will continue to be useful.

A major responsibility of the military services is to exercise careful stewardship of the potentially hazardous materials that are critical to the successful execution of the national security mission. We are required, morally and legally, to prevent contamination of the environment and to assure the well-being of the general population. Of course, the military services must comply with the ever-increasing federal and state regulations. More important, we must apply preventive medicine to protect our own workers on base and civilians outside the base from adverse effects of accidental or occupational exposure to hazardous materials. For preventive medicine to be effective, we must understand and evaluate the health hazards peculiar to each substance proposed for use. A chemical's effect on the human body must be reliably predicted. Its chemical and physiological properties must be investigated and the ways in which the chemical is absorbed, distributed, detoxified, and excreted must be known. In addition, the nature and severity of detrimental or toxic effects must be established. Once this enormous data base has been developed for the chemical of concern, then tolerance criteria can be established for personnel who handle or become exposed to these chemicals. Special handling, shipping and storage techniques can be determined, protective clothing and gear can

be developed and appropriately utilized, and therapeutic methodologies established. From an Air Force viewpoint, the data base resulting from our research on AF-unique materials, when properly time-phased with the development of weapon systems, will insure the safe deployment of these systems with respect to the general public as well as Air Force personnel. An excellent example of this is the application of our hydrazine toxicology data to the establishment of operational guidelines for the hydrazine-fueled emergency power unit used in the F-16 aircraft. A wide range of toxic response data (ranging from acute inhalation studies in the 1961-67 time frame to metabolism/kinetics/skin absorption studies conducted in the 1979-82 time frame) was available for F-16 systems program office planning. The SPO, assisted by AMRL scientists, applied this to the pertinent operational scenarios to produce guidelines for insuring ground air crew safety through appropriate procedures, monitoring. personal protection, and medical surveillance.

For the Air Force, development of the toxicology data base is the task of the scientists at the Air Force's Toxic Hazards Division at Wright-Patterson AFB Ohio. Their notable success to date has been further enhanced by joint work with the US Navy. The Navy detachment at Wright-Patterson helps support the Toxic Hazards Research Unit and also has its own inhouse toxicology effort.

In my opinion, there are two major challenges facing researchers in environmental toxicology today. The first of these relates to the logistics of conventional toxicology research. You know better than I the problem of the exposure of large numbers of many species of laboratory animals for long periods of time to precisely controlled doses of potentially toxic substances. Federal and state laws and regulations potentially force the study of virtually endless numbers of these substances. Resources and facilities to meet this requirement clearly are inadequate. Already you are called upon frequently to provide ad hoc guidance for the employment of substances or for the evaluation of the risk of past exposures to materials for which the data base is inadequate.

We must somehow find the means to reduce the time and cost of research in toxicology. In my opinion, the most promising avenue is in scientific modeling, such as toxicokinetics. Hopefully, methods can be validated which will allow a much more limited data-gathering effort to be successfully predictive of the deleterious effects of classes of materials on humans and on ecologic systems.

The second problem is the growing effectiveness of groups which oppose the use of animals in life sciences research of any kind. Last year's crop of congressional legislation to limit animal research failed because it was proposed that a segment of each federal research appropriation in which animal models were used be set aside specifically to develop alternate research methods. The bills so far proposed for the next session of the congress are not

yet tied to specific budgetary restrictions, but rather are written to establish a federal policy discouraging the use of animals for research. That will be a most important first step in the campaign to eliminate the only means available now to meet the needs of society in many aspects of medical, pharmaceutical, toxicological, and environmental research.

Obviously, a successful solution to the first problem, the time and resources cost of conventional toxicologic research, will reduce, but probably never eliminate, the need for animal research. I encourage your attention to these two concerns. You will hear several Air Force and Navy scientists present papers at this conference. From a scientific methodology standpoint, the toxicology studies conducted by these scientists are little different from those conducted by you from industry and academia. Therefore, an exchange of knowledge and ideas at this meeting will benefit both the civilian and military communities. Over the years this conference has been unique. By size and design it has been a forum in which the personal participation of every attendee is encouraged. This year's conference is no different. I strongly encourage each one of you to contribute what you can to the discussions. By doing this, I am confident we will all benefit greatly from what promises to be one of our finest meetings.

SESSION I

AN OVERVIEW OF IMMUNOTOXICOLOGY METHODOLOGY

Chairman

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PAPER NO. 1

ASSAYS FOR CELL-MEDIATED AND HUMORAL IMMUNITY

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The subjects of assays of cell-mediated immunity (CMI) and humoral immunity are far too large to cover adequately in 20 minutes. I will therefore focus on assay systems in most general use and will forego lengthy descriptions of assays which I am sure subsequent speakers will cover.

Although related, the cell-mediated immune response and humoral immune response are frequently treated as separate systems. Originally the distinction was made based on the following criteria:

1. Cell-mediated immune functions are carried out by cells (mainly T-cells and macrophages). Immunity may be transferred to another animal by transfer of specifically sensitized cells, but not blood serum.

2. Humoral immunity is conferred by specific antibody. The immunity of one animal may be transferred to a non-immune animal by passive transfer of serum (antibody), but not cells.

Assays for cell-mediated immune function may be broken down into in vivo and in vitro categories. Commonly used in vivo assays are listed in Table 1. Delayed hypersensitivity responses (DHR) are an excellent means of assessing CMI function. The method is detailed by Luster et al. (1982). Toxicant contact with the immune system may be initiated at any time prior to sensitization and maintained until animal sacrifice. Graft vs host and skin graft rejection assays are based on recognition of non-self histocompatibility antigens by the animal. These assays are not used to the same extent as DHR systems owing to inherent technical difficulties and poorer quantitative aspects. Tumor cell killing assays are frequently used to assess CMI (Dean et al., 1982). These systems utilize transplantable tumors as the foreign antigen, and the assessment parameter, tumor cell killing, is obviously a practical endpoint. The basis of the three assays is the same: T-lymphocytes respond to specific foreign stimuli (either foreign environmental antigens or histocompatibility antigens) and either directly (via

cytotoxic T-cells) or indirectly (via lymphokines which attract and activate scavenger cells) to eliminate the foreign substance. Cell mediated immunity is the most conservative means to deal with intrusion of a foreign substance in that it is dealt with locally.

TABLE 1. ASSAYS FOR CELL-MEDIATED IMMUNITY IN VIVO

1. Delayed Hypersensitivity Responses (Antigens)
2. Skin Graft Rejection (Histocompatibility)
3. Graft vs Host Reactions (Histocompatibility)
4. Killing of Transplantable Tumors

In vitro assays for CMI measurement are numerous; some of the most commonly used systems are listed in Table 2. Lymphocyte proliferation assays are most often used. The measurement of proliferation is accomplished by determining incorporation of a radiolabeled DNA precursor (usually ^{3}H-thymidine) into DNA. Proliferation can be triggered in T-lymphocytes by specific mitogens (concanavalin A or phytohemagglutinin), antigens (very low response), or by mixing lymphocytes from the chemical-treated animal with proliferation-arrested lymphocytes from an animal with different histocompatibility antigens. Although a sensitive index of cell damage, proliferation per se is not actually a functional assay of lymphocyte status. Numerous investigators have demonstrated that lymphocytes can function (produce their end product effector chemicals, lymphokines) in the absence of proliferation. Lymphokine assays, then, may be a better indicator of actual lymphocyte function. Of the three lymphokines listed [migration inhibitory factor (MIF), gamma interferon, and interleukin 2 (IL2)], gamma interferon has several noteworthy advantages. It is highly quantifiable, the unit of biological activity is well-defined, and an internationally recognized standard is available such that inter-laboratory validation of data is possible (Archer et al., 1980). The aforementioned assay systems test the status of effector cells in CMI. The responder cells, polymorphonuclear leukocytes and macrophages, can also be assessed for functionality by testing their phagocytic capability (easiest to do), their response to chemotactic stimulators, and their cytostatic action on tumor cells.

TABLE 2. ASSAYS FOR CELL-MEDIATED IMMUNITY IN VITRO

1.	Lymphocyte Proliferation	A. Mitogens B. Antigens C. MLR
2.	Lymphokine Assays	A. MIF B. IFN (gamma) C. IL2
3.	PMN and Macrophage Function	A. Phagocytosis B. Chemotaxis C. Cytostasis

As previously mentioned, the CMI system is the most conservative means by which an animal can deal with intrusion of a foreign substance. If the CMI system is overwhelmed by simply too much of the foreign material, the regional lymph nodes and ultimately the humoral immune response become involved.

Humoral Immunity: To reiterate, the basis of humoral immunity is the production of antibody which specifically reacts with the eliciting antigen (foreign substance). Antibody facilitates removal of the foreign substance by increasing its size (agglutination or precipitation), by coating foreign organisms (opsonization) which facilitates phagocytosis, and by binding to antigen with subsequent complement activation.

Methods to quantitate humoral immunity are numerous (Table 3). Serum immunoglobulin levels, while revealing, are relatively insensitive. It is more desirable to monitor antigenically induced antibody. It is most desirable to monitor a primary response (animal's first encounter with antigen) and a recall response (animal's response to an antigen it has encountered before). The choice of antigens is also important. Representatives of thymus-dependent and the two classes of thymus-independent antigens should be included for a thorough study.

TABLE 3. ASSAYS FOR HUMORAL IMMUNITY IN VIVO

1. Serum Immunoglobulin Levels
2. Radioimmunoassay or ELISA
3. Hemolytic Plaque Assays
4. Hemagglutination Assays

In vivo assays in current use include radioimmunoassay (RIA), enzyme-linked immunosorbent assays (ELISA), and hemolytic plaque assays. RIA and ELISA can monitor total immunoglobulin production, or specific immunoglobulin production in response to antigen. The data are quantitative and the assays can be automated. From the data, one cannot discern between some loss of response by all plasma cells, or total loss of antibody production by some plasma cells. Hemolytic plaque assays can partially answer this question, as antibody-producing plasma cells are visualized directly. ELISA was recently reviewed by Vos et al. (1982), and hemolytic plaque assays by Munson et al. (1982).

Subsequent papers will give more detailed information about how these systems are applied and the types of resultant data.

It must be said, in conclusion, that although these two major arms of the immune response are vitally important to the animal's well-being, these tests do not cover the full spectrum of the immune system. One glaring example of a deficiency would be the secretory immune system, which is of vital importance to the animal, as the

first lymphoid tissue to come in contact with many ingested substances (and in the highest doses), and for which no reliable assay system exists.

The question often arises as to which approach, in vitro or in vivo, is best. It has been our experience that in vitro assays are valuable adjuncts in discovering underlying mechanisms but that they may not always be predictive of ultimate in vivo consequences. Clearly, the most meaningful data are obtained from in vivo testing. Some of the reasons for this statement are detailed in Table 4.

TABLE 4. ADVANTAGES OF IN VIVO ASSAYS

1. Distribution and Metabolism Accounted For
2. Most Realistic Situation
3. Can be Correlated with Host Resistance Assays

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PAPER NO. 2

USE OF INTERFERON SYSTEMS IN IMMUNOTOXICOLOGY

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INTRODUCTION

Interferons are a group of proteins that were originally described as being produced in response to virus infections and acting by making virgin cells refractory to virus infection[1,2]. It has recently become apparent that interferons are much more complex than originally as described[3,4]. There are now several types of interferons[5]. Alpha interferon is primarily produced by leukocytes in response to virus or double-stranded RNA-(polyriboinosinic-polyribocytidylic acid=poly I:C). Beta interferon is primarily produced by fibroblasts in response to virus or poly I:C. Therefore, unless purified, interferon produced by fibroblasts after poly I:C challenge contains a minor alpha component and a major beta component. Gamma (type II immune) interferon is produced as a lymphokine as part of an immune response of lymphoid cells to antigen or mitogen. Alpha and beta interferons appear to have some structural and functional similarities but gamma interferon appears to be a distinct entity[6].

Interferons have also been shown to have several additional activities in addition to the originally described antiviral activity[3,4]. These include regulation of cell growth and division, regulation of immune responses, and cytotoxicity to tumors and tumor cells[3,4]. As a result of these activities, the use of interferons in clinical anti-cancer trials is now being actively pursued[7].

Since interferons may be involved in defenses against tumors, it was of interest to study the interactions between interferons and carcinogens. Several workers had suggested that carcinogens could inhibit the induction of interferon, while closely matched, poorly, or non-carcinogenic analogues had no effect on interferon induction[8-12]. We began a survey of the effects of a wide

variety of types of carcinogens and analogues on interferon induction[13-20]. Our results to date suggest a strong correlation between carcinogenic potential of a chemical and the effects of the chemical on interferon induction[13-20].

MATERIALS AND METHODS

MOUSE EMBRYO FIBROBLAST CULTURES

C3H/He mice were originally obtained from Laboratory Supply, Indianapolis, Indiana and then maintained and bred in our laboratory. Fifteen to 18 day-old embryos were surgically removed from pregnant dams, and then trypsinized to single cells. The cells were next suspended in Gibco (Grand Island, New York) minimal essential medium with 10% fetal bovine serum. Second or third passage cultures were used in all experiments and were plated in 25 cm^2 plastic tissue culture flasks. Cultures were immediately used after reaching confluency[13-19].

MOUSE SPLEEN CELL CULTURES

Female 6-8 week old Swiss/Webster mice were obtained from Laboratory Supply Company, Indianapolis, Indiana. Cultures of 5 x 10^6 spleen cells were prepared in 1 ml of RPMI-1640 medium (Gibco, Grand Island, New York) supplemented with 1% fetal bovine serum and 5 x 10^{-5}M 2-mercaptoethanol[20].

CELL VIABILITY DETERMINATION

Cell viabilities were determined by trypan blue dye exclusion[13-20].

PRODUCTION OF ALPHA/BETA INTERFERON

Alpha/Beta interferon was induced in mouse embryo fibroblast cultures by stimulating with 50 μg of poly I:C for 60-90 minutes, and then adding additional fresh tissue culture medium. In some cases, Newcastle disease virus was the inducer. DEAE-dextran was included to insure maximum interferon production. Tissue culture supernatants were harvested at 24 hours and assayed for interferon antiviral activity[13-19].

PRODUCTION OF GAMMA INTERFERON

Mouse spleen cell cultures were stimulated with 34 μg of phytohemagglutinin-P and incubated for 3 days at 37°C in 5% CO_2. Culture supernatants were then harvested and assayed for interferon activity[20].

INTERFERON ASSAY

Antiviral titers were measured by performing a plaque reduction assay on mouse L-929 cells with the Indiana strain of vesicular stomatitis virus as the test virus[21]. The antiviral titer corresponded to the reciprocal of the furthest dilution of test sample that reduced virus plaques by 50%. In this assay, one interferon antiviral unit is equivalent to 0.88 NIH G-002-904-511 reference units.

STATISTICAL ANALYSIS

The data were analyzed by means of Student's t-test. P values of <0.05 were required for statistical significance.

RESULTS

EFFECTS OF CHEMICALS ON ALPHA/BETA INTERFERON

Mouse embryo fibroblasts were pretreated with chemicals and then challenged to induce alpha/beta interferon as described in the protocol in Figure 1. Several pairs of different types of probable carcinogens and poor or non-carcinogens were included. In all cases except two, benzidine and diethylstilbestrol, carcinogen pretreatment significantly decreased alpha/beta interferon production (Table 1)[13-19]. Since the interferon assay is a titration, a statistically significant decrease of 50% or greater as compared to a solvent-only control was required. Poor or non-carcinogens had no effect on alpha/beta interferon induction (Table 1)[13-19]. No effect on viability was observed after any of the chemical treatments.

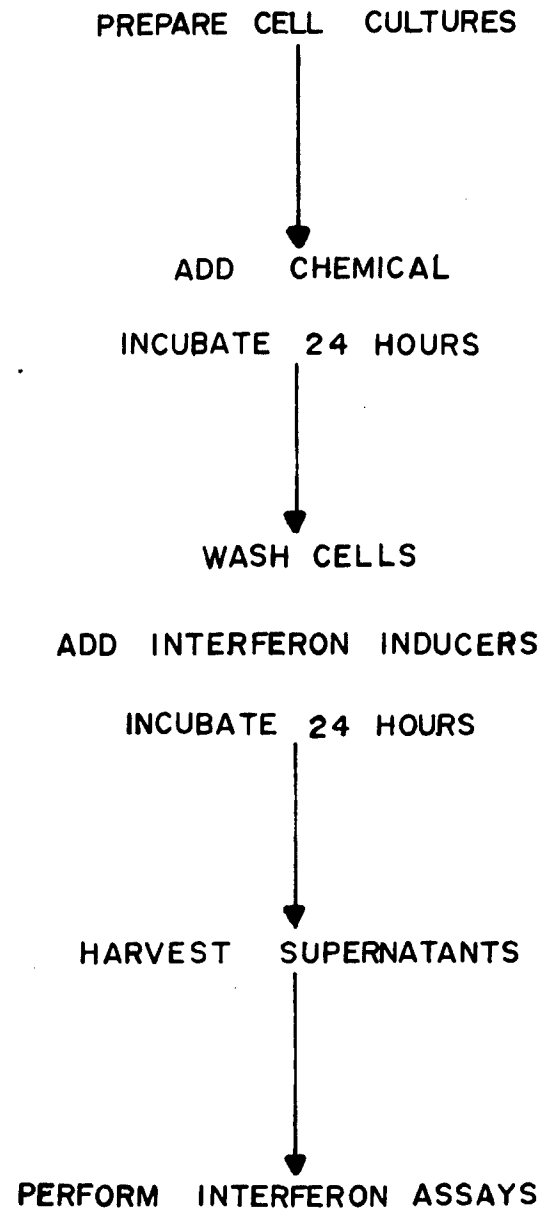

Figure 1. Protocol for determination of the effects of carcinogens on interferon induction.

EFFECTS OF CHEMICALS ON GAMMA INTERFERON

7,12-dimethylbenz-(a) anthracene (DMBA) was added to spleen cell cultures prior to induction of gamma interferon. Gamma interferon production was significantly inhibited, but in this case viability was slightly reduced (data not shown)[20]. When DMBA was added to target L-929 cells together with exogenous gamma interferon, no effect on the antiviral activity of the gamma interferon was observed.

TABLE 1. EFFECTS OF CARCINOGENS AND ANALOGUES ON INTERFERON INDUCTION

Chemical	In vivo[a] Carcinogenic Potential	Effect on Interferon Induction	Minimal Effective Concentration
β-Propriolacetone	+	NS[b]	--
γ-Butyrolacetone	-	-52%	100 μM
Pyrene	-	NS	--
Benzo-(a)-pyrene	+	-90%	0.05 μM
1-naphthylamine	-	NS	--
2-naphthylamine	+	-56%	100 μM
Anthracene	-?	NS	--
7,12-dimethylbenz (a) anthracene	+	-86%	4 μM
9,10-dimethylanthracene	+?	-50%	100 μM
Chloroethanol	-	NS	--
Chloroacetic acid	-	NS	--
Chloroacetaldehyde	+	-77%	0.005 μM
Ethyl methanesulfonate	-?	NS	--
Methyl methanesulfonate	+	-91%	0.05 μM
1,1,1-Trichloroethane	-	NS	--
Chloroform	+	-54%	100 μM
Amorphous Nickel Sulfide	-?	NS	--
Crystalline Nickel Sulfide	+?	-75%	2 μg/ml
3,3'-5,5'-tetramethylbenzidine	-	NS	--
Benzidine	+	NS	--
2-aminofluorine	+	-81%	0.005 μM
Aflatoxin B_1	+	-93%	0.05 μM
Number 4 fraction tobacco smoke condensate	+	89%	10x[c]
Sytrene oxide	+?	-90%	0.05 μM
2-methylquinoline	+?	-79%	0.01 μM
4-aminobiphenyl	+	-73%	0.01 μM
Hydrazine sulfate	+	-72%	0.01 μM
Aniline-HCl	+	-75%	0.01 μM
Diethylstilbestrol	+?	NS	--
Isobutyl Nitrite	+?	61%	0.01%
"RUSH" (Recreational isobutylnitrite)	+?	62%	0.01%
Ascorbic Acid	-	NS	--
Glycine ▼ Leucine	-?	NS	--
Glycine ▼ Isoleucine	-?	NS	--

[a] Data from references 13, 14, and 22.
[b] NS = Not a significant decrease.
[c] Arbitrary laboratory concentrations.

DISCUSSION

Pretreatment of mouse embryo fibroblasts with a variety of carcinogens resulted in significant depression of alpha/beta interferon induction by poly I:C or Newcastle disease virus[13-19]. Treatment of the cells with poorly or non-carcinogenic analogues had no significant effect on interferon induction.

Of the 34 chemicals tested, only the following were exceptions: Benzidine, a carcinogen, had no effect on interferon induction. This may be due to insufficient activation of benzidine by the mouse embryo fibroblasts[13]. Diethylstilbestrol (DES) also had no effect on interferon induction. The lack of effect of DES may have been due to the apparent unique mechanism of carcinogenic action of this chemical. DES appears to act through an hormonal action and is not usually mutagenic to bacteria[17,23].

Gamma interferon induction was also inhibited by carcinogens[20]. Since many different types of carcinogens apparently affect the production of different types of interferons, it is possible that multiple mechanisms may have been involved. Viability of the cell cultures was not dramatically affected, and virus replication was not depressed by carcinogen-treatment[9]. Therefore, it is unlikely that carcinogen treatment resulted in a non-specific general toxic shutdown of cellular metabolism. Other induced proteins in addition to interferon may also be affected.

Since carcinogen treatment had no effect on preformed exogenous interferon[20], it is unlikely that the carcinogens were bound to the interferon inactivating it. Rather, it is likely the carcinogens affected the actual interferon production mechanism. The carcinogens may be binding to cellular nucleic acids, preventing the production of interferon.

The results of the present study suggest a high correlation between carcinogenic potential of a chemical and suppression of interferon induction. After extensive further testing, inhibition of interferon induction may prove useful as part of a battery of screening tests for carcinogenic potential of chemicals. This system has the advantages of using fibroblasts, which may be primary target cells for carcinogens in vivo, and being relatively straightforward to interpret. As assays for interferon improve with the development of radioimmune assays and enzyme linked-immunoassays, inhibition of interferon assays may be readily performed in a number of laboratories.

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PAPER NO. 3

APPLICATION OF FLOW CYTOMETRIC METHODS IN IMMUNOTOXICOLOGY

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INTRODUCTION

Flow cytometry is an instrumental technique that permits the simultaneous, quantitative measurement of a variety of properties on individual cells in a flow stream at rates of several thousand cells per minute. In addition, many instruments provide the capability to sort cells on the basis of preselected determinants. Examples of parameters that can presently be measured by commercial instruments include: single or dual wavelength fluorescence, low or 90° angle light scatter, cell number, cell size or volume, and others. Fluorescence provides the basis for the majority of applications and has been utilized in the development of methods for the analysis of DNA cell cycle phase, cell surface marker expression, phagocytosis and viability in suspensions of single cells. These analytical techniques make possible the study of chemically-induced alterations in cell proliferation and differentiation, specific cell functions, such as phagocytosis and cytotoxicity, and the identification of specific cell populations.

METHODS

INSTRUMENTATION

The design of the flow cytometer is similar to that of a fluorometer, the three primary components being a source of excitation (usually a laser), a means of sample containment (flow stream), and a detector(s). A schematic diagram is presented in Figure 1. A suspension of single cells is introduced into a stream by means of a laminar flow chamber adjusted so that the cells pass through in single file. A highly efficient excitation source is required in order to measure fluorescence on individual cells. This is achieved in most instruments using a tunable laser light source, although some instruments employ a mercury arc lamp. Fluorescence detection is accomplished by placing photoelectric detectors perpendicular to the exciting light beam. Specificity is obtained by using a combination of a monochromatic light source and appropriate barrier filters. Tunable argon or krypton lasers are readily available and

provide a variety of useful excitation wavelengths. A feature that discriminates cell sorters from simple cytofluorometers is the ability to separate cells on the basis of predetermined analytical criteria. This is accomplished by vibrating the sample stream at a given frequency to break it into droplets containing individual cells at a measureable distance after analysis has occurred. When the cell satisfies predetermined sort criteria, a charge is placed on the droplet containing that cell which is then deflected either to the left or the right by a pair of deflecting plates.

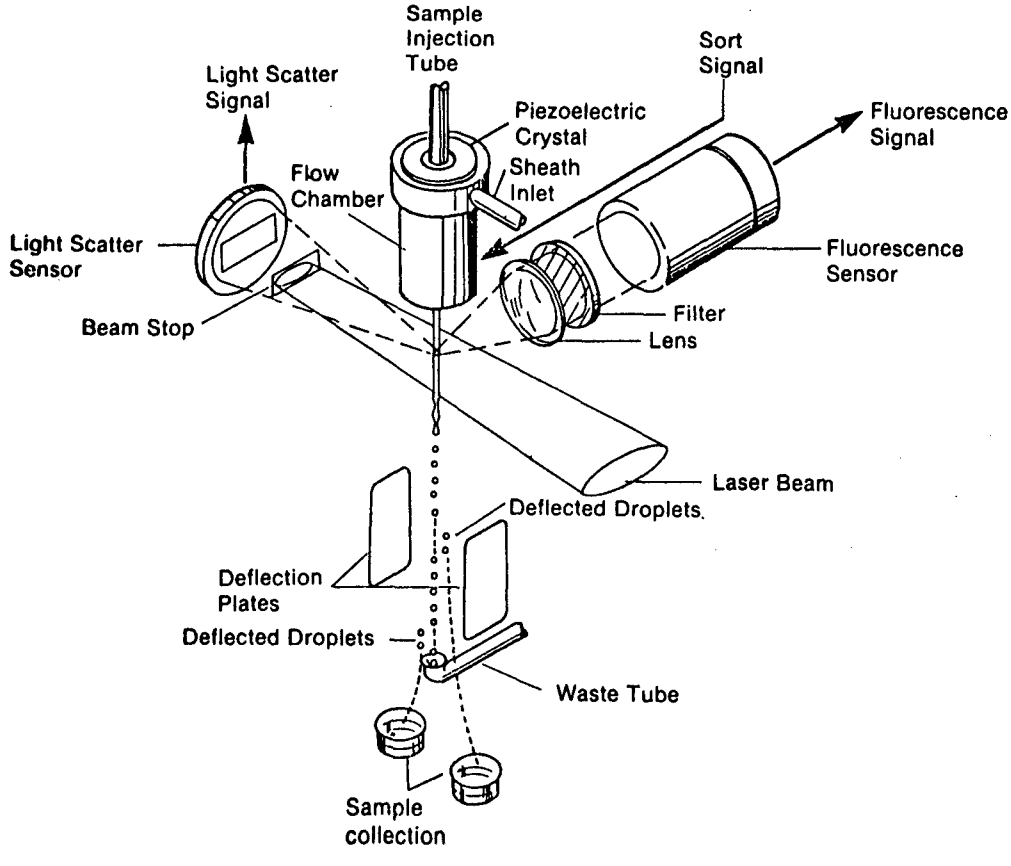


Figure 1. Schematic diagram of a laser based cell sorter (flow cytometer). (Courtesy Coulter Electronics, Hialeah, Florida.)

PARAMETERS

LIGHT SCATTER

When a cell or particle passes through a beam of coherent polarized light (laser beam), light is scattered in all directions. Low angle light scatter or light scattered in the forward direction (0.5-20°) is largely due to diffraction from biologic membranes and under certain circumstances can be used to discriminate cells on the

basis of size. Refractive index also contributes to low angle light scatter, and the measurement is most often used to discriminate between live and dead cells. Right angle light scatter (90°) is largely dependent on internal structures. By measuring low and 90° angle light scatter simultaneously it is possible to distinguish erythrocytes, granulocytes, lymphocytes and macrophages in whole blood. We have used this technique to monitor cell populations in bone marrow as well (Figure 2).

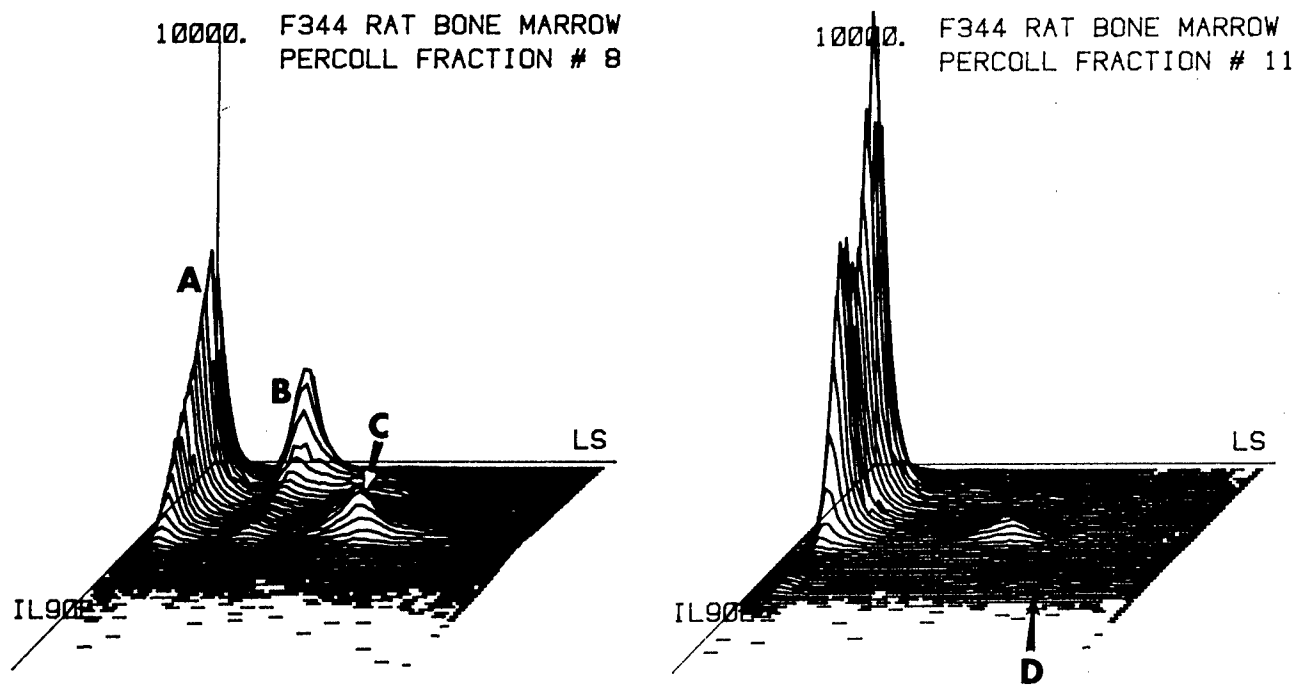


Figure 2. Three dimensional projections of low angle light scatter (LS) versus 90° angle light scatter (IL90) versus cell number for F-344 rat bone marrow cells fractionated on Percoll® (Pharmacia Fine Chemicals) gradients. A - Red Blood Cells; B - Lymphoid Cells; C - Myelocytic and Monocytic Precursor Cells; D - Megakaryocytes and Granulocytes.

FLUORESCENCE

Fluorescence detection methods are predicated on the ability of a fluorescent substance to be taken up and/or bind to specific structures on or within the cell. Analysis of DNA content in individual cells is based on the use of fluorescent compounds that stoichiometrically bind to DNA and for which the intensity of fluorescence is proportional to DNA content. Under these conditions, fluorescence intensity can be used to quantitate the proportion of the total cell population in each phase of the cell cycle. Examples of staining reagents that have been used for this purpose include

the interchelating dyes, ethidium bromide and propidium iodide, and the "groove binding" dyes, mithramycine and chromomycin. Cells must be fixed in order to allow entry of the latter substances as well as fluorescein into the cell, and is the basis for a number of live-dead cell assays.

Immunofluorescence can be used to identify and quantitate cells on the basis of surface markers or membrane antigens. Antibodies directed against surface immunoglobulins or alloantigens can be used to discriminate between cell types (e.g., B and T lymphocytes) and to monitor differentiation in these populations. The use of two fluorochromes, combined with the recent commercial availability of dual laser systems, provides the capability to discriminate cells on the basis of several determinants, simultaneously.

MISCELLANEOUS

A number of additional parameters or techniques have been theoretically possible for some time and have recently become commercially available. These include: time-of-flight, coulter volume, slit scan, and fluorescence anisotropy. In addition to direct measurements of cell size or volume, these techniques allow for analysis of particle shape functions (time-of-flight), cell surface marker distribution (slit scan), and membrane fluidity (fluorescence anisotropy).

Detailed descriptions of cytofluorometric instrumentation and its applications in biology have been presented elsewhere (Horan and Wheeless, 1977; Melamed et al., 1979; Irons, 1981).

APPLICATIONS IN IMMUNOTOXICOLOGY

CELL CYCLE ANALYSIS

The cell cycle can be divided into four phases on the basis of DNA synthesis: G1, the first gap or period of no measureable DNA synthesis; S, the period of active DNA synthesis and replication; followed by G2, the second gap during which DNA synthesis is complete and the DNA content of the cell is twice the diploid content found in G1; and M, or mitosis. Additional compartments, such as Go, can be considered on the basis of RNA or protein synthesis; however, analysis of G1, S, and G2/M phase distributions enables the study of growth kinetics in a given tissue or population of cells. Quantitation of the DNA content of individual cells provides a means to determine in what phase of the DNA cycle they reside. Normal bone marrow and stimulated cells in culture contain cells undergoing asynchronous exponential growth. Asynchrony is defined by two characteristics of growing cells, namely, that they move through the cell cycle independent of one another and that the transit time for any phase is independent of the transit time for any other phase.

Therefore, at any given point in time, the probability that a cycling cell will be in a particular phase of the cell cycle is proportional to the amount of the total cell cycle transit time occupied by that phase. In a theoretical asynchronous population, the distribution of DNA content among cells consists of a single absolute value for G1/Go, a linear continuum of values for DNA content in S phase, and a single value for G2/M that is exactly twice that of G1/Go. An actual DNA distribution histogram is presented in Figure 3A and illustrates the contribution of experimental error, both instrumental and biological. The single values theoretically described for Go/G1 and G2/M are replaced by gaussian curves. Pretreatment of the animal with a cycle specific agent results in a partial synchronization, in this case accompanied by an accumulation of cells in G2/M (Figure 3B). Quantitative compartmental analysis of DNA histograms can become extremely complex, and several computer based methods exist for parametric as well as non-parametric histogram analysis. We have previously used this approach to study the effects of repeated benzene administration on cell cycle kinetics in rabbit and rat bone marrow (Irons et al., 1979; Horan et al., 1980; Muirhead et al., 1980).

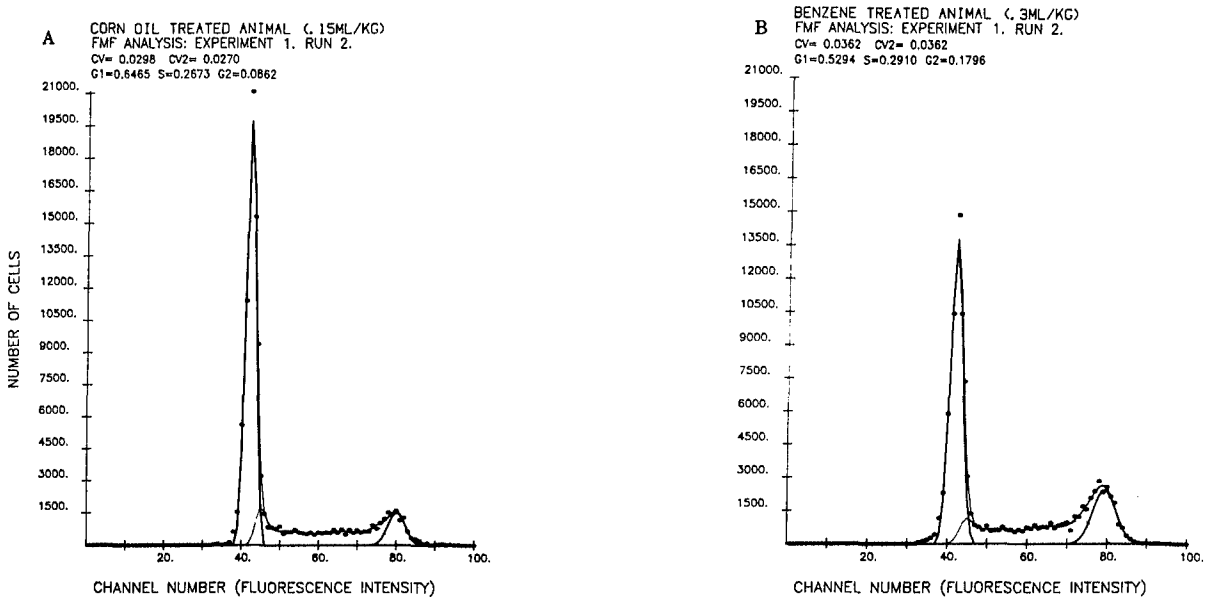


Figure 3. Two dimensional histograms of DNA content in bone marrow cells of (A) control and (B) benzene treated NZW rabbits. Corn oil or benzene in corn oil was administered daily for three days. CV, Coefficient of variation for G1 and G2 peaks, respectively. Values for G1, S, and G2 are the fraction of total cells in each compartment. (Mithramycin stained.)

ANALYSIS OF MACROPHAGE FUNCTION

CYTOSTASIS

A parameter often used to measure macrophage function is target cell cytostasis or cytotoxicity. This is usually monitored by measuring the uptake of ^{3}H-thymidine into tumor target cells or determining the effect of activated macrophage populations on this process. We have recently applied quantitative DNA cell cycle analysis for this purpose and have found it to exceed conventional tracer methodology in both sensitivity and resolution. Murine macrophages were activated by the eliciting agents BCG or maleic anhydride vinyl ether (MVE-2) as previously described (Dean et al., 1978) and resident peritoneal macrophages harvested and cultured with a leukemia cell line (MBL-2). Cytostasis of leukemia target cells, as measured by ^{3}H-thymidine incorporation, was significantly depressed only after 48 hours, whereas cell cycle analysis proved sensitive enough to monitor changes as early as 12 hours. Furthermore, cell cycle analysis revealed that macrophages activated with different eliciting agents altered the kinetics of cytostasis in culture (Table 1).

TABLE 1. ASSESSMENT OF MACROPHAGE FUNCTION USING LEUKEMIA TARGET CELL CYTOSTASIS[1]

Incubation Time (Hr)	Percent of Leukemia Target Cells in S Phase[2]			
	MBL-2 Cells Alone	Macrophages		
		Control	BCG-	MVE-2-Elicited
12	----	21.5	0.0	21.6
18	42.6	29.8	6.5	10.7
24	64.2	37.5	0.0	0.0
48	64.2	69.8	23.0	12.7
	^{3}H-THYMIDINE INCORPORATION (DPM)			
48	38,599	27,673	11,601	15,992

1 Resident peritoneal macrophages were harvested from untreated B6C3F1 mice or mice previously injected with BCG (14 days previous) or MVE-2 (6 days previous) and purified by adherence to plastic microexudate flasks.

2 Based on counts of 20,000 target cells/culture.

PHAGOCYTOSIS

The ability of macrophages to phagocytize fluorescein labeled plastic microspheres is also a measure of macrophage function and can be assessed using cytofluorometric methods (Figure 4). This

technique not only provides a means to enumerate phagocytic cells but also the capability to quantitate phagocytosis on an individual cell basis. This technique can be applied to the assessment of phagocytosis both in vivo as well as in vitro.

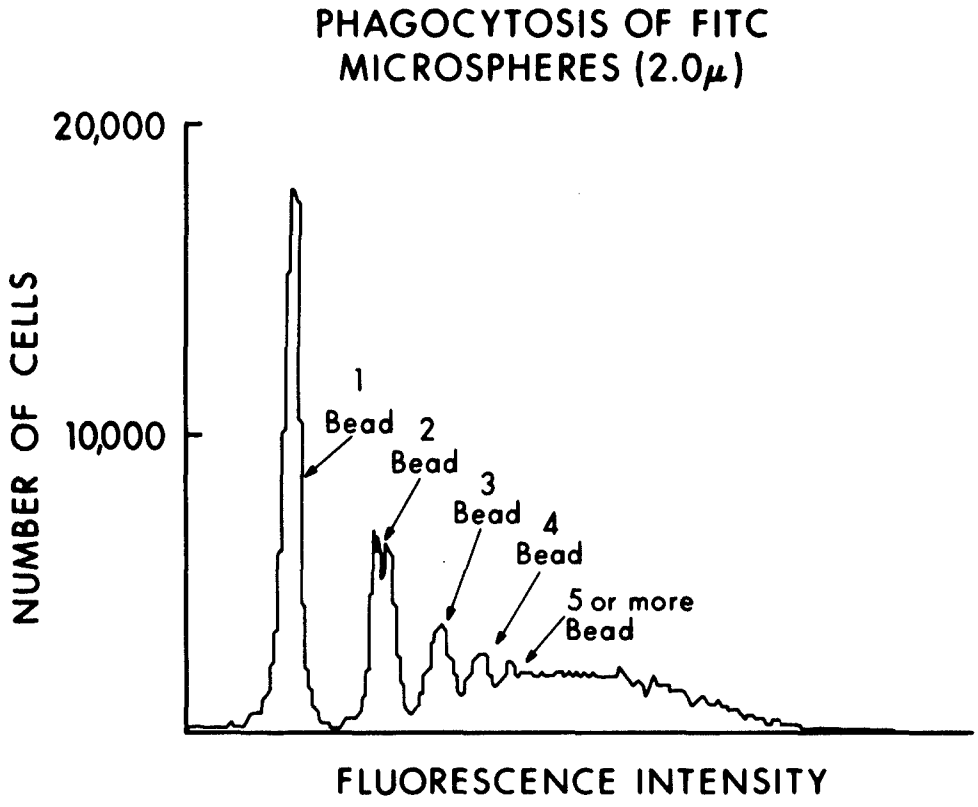
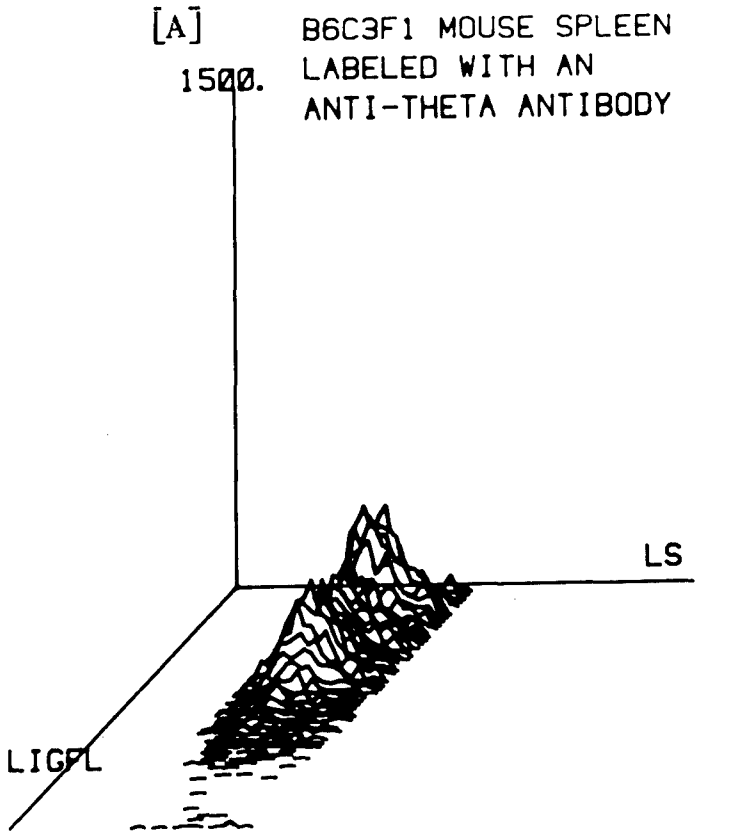


Figure 4. Quantitative cytofluorometric analysis of macrophage phagocytosis using fluorescein isothiocyanite conjugated Covaspheres© (Covalent Technology).

CELL SURFACE MARKER ANALYSIS

Cytofluorometric analysis of cell surface antigens has become an important immunologic technique for the identification of lymphocyte subpopulations and in the study of lymphocyte differentiation. It can be used to quantitate surface marker expression on individual cells as well as enumerate cells bearing particular markers indicative of cell type or level of differentiation. For example, T and B lymphocytes can be enumerated using fluorescent antibodies directed against theta antigen (Figure 5a) or surface immunoglobulin (Figure 5b), respectively. In addition, expression of surface alloantigens accompanying functional differentiation, such as those expressed by T helper or suppressor lymphocyte subpopulations, can be employed to assess lymphocyte differentiation in vivo and in vitro by quantitating the number of cells expressing differentiation markers in culture or in tissue under controlled conditions (Irons et al., 1981).



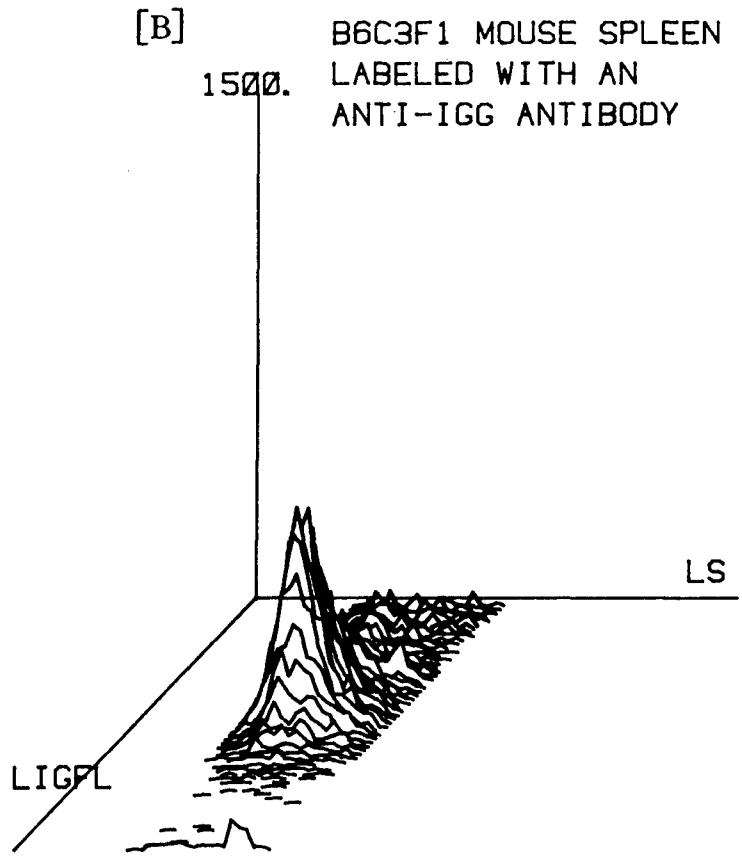


Figure 5. Examples of cytofluorometric analysis of T and B lymphocytes in B6C3F1 mouse spleen.

CONCLUSION

Cytofluorometry provides a sensitive and powerful tool that is being widely exploited in cell biology in general and immunobiology in particular. It offers the advantage of measuring a wide variety of parameters on individual cells. Although flow cytometry has only recently been applied to the field of toxicology, its use is limited only by the imagination of the investigator and should greatly facilitate the evaluation of the effects of chemicals on such phenomena as cell growth, differentiation, and function.

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PAPER NO. 4

HOST RESISTANCE MODELS AVAILABLE FOR IMMUNOTOXICOLOGIC STUDIES

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INTRODUCTION

For about 25 years various investigators at IIT Research Institute (IITRI) have been developing animal model systems to measure the holistic response to drugs, chemicals, and air pollutants. In 1958, the first report on the effects of ozone (O_3) on bacterial pneumonia was published by Miller and Ehrlich. That research, supported by the United States Air Force, was the basis for our subsequent programs in which several infectious disease models were used to evaluate the effects of a variety of toxicants. Table 1 shows thirteen infectious disease models and one tumor challenge model we have used in mice, hamsters, guinea pigs, or squirrel monkeys. With the exception of the diethylstilbestrol (DES), all toxicants were administered by the inhalation route. Other investigators are working with a variety of tumor and infectious disease models to evaluate toxicants administered by various routes and Dean et al. (1982b) have summarized this research. As a consequence there is a wide selection of host resistance models available for use.

Recently, increasing attention has been focused on the two-directional aspect of immune modulation following exposure to the various test agents. First, it has been aimed towards detection and prevention of adverse effects of agents on host immunity, with a view towards avoiding a resulting increased susceptibility to infectious disease or to a malignant process. Secondly, attention has been directed toward the stimulation of host resistance to certain disease entities.

To develop an immunologic profile, the selection of the appropriate assay system (Table 2) is dependent upon which aspect of immune modulation is being studied, i.e., suppression or stimulation; reproducibility of the disease state; susceptibility of a given host to a particular disease; easily measured parameters; route of exposure to both the infectious organism/tumor system and test material; and the cost. Host resistance assays are important since they reflect in the host the net result of alterations in specific immune aspects that may be detected separately by predominantly in vitro assays.

TABLE 1. ANIMAL MODEL SYSTEMS ESTABLISHED AT IITRI

Toxicant	Organism	Animal Model	Reference
O_3	Klebsiella	Mice, hamsters	Miller & Ehrlich 1958
NO_2	Klebsiella Klebsiella	Mice Squirrel monkeys	Ehrlich, 1963 Ehrlich, 1966
Cigarette Smoke	Klebsiella Staphylococcus Diplococcus Influenza	Mice	Spurgash et al., 1968
NO_2	Klebsiella Influenza	Squirrel monkeys	Henry et al., 1970a
NO_2 and Cigarette Smoke	Klebsiella	Hamsters	Henry et al., 1970b
Hyperoxia	Influenza	Mice	Fenters et al., 1975a
PtO_2 and PdO	Klebsiella Influenza Streptococcus	Mice	Fenters et al., 1975b
MnO_2	Klebsiella Influenza	Mice	Maigetter et al., 1976
NO_2/O_3 Mixtures	Streptococcus	Mice	Ehrlich et al., 1977
Sulfates	Streptococcus	Mice	Ehrlich et al., 1978
SO_2 and Nitrates	Streptococcus	Mice	Ehrlich, 1979
Acid mists	Influenza	Mice	Fenters et al., 1979
O_3	Mycobacterium	Mice, guinea pigs	Thomas et al., 1981a
PAN	Streptococcus Mycobacterium	Mice	Thomas et al., 1981b
Industrial Particulates	Streptococcus	Mice	Aranyi et al., 1981a
As_2O_3	Streptococcus	Mice	Aranyi et al., 1981b
DES	Listeria, Herpes, Trichinella, Influenza, Streptococcus B16-F10 melanoma	Mice	Bradof et al., 1982

TABLE 2. SELECTION OF HOST RESISTANCE MODEL

- Immunomodulation
  - Suppression
  - Stimulation
- Cost
- Reproducibility of Disease State
- Susceptibility to Disease
- Easily Measured Parameters
- Route of Exposure
  - Test Agent
  - Test Material

This paper stresses the importance of selecting the appropriate assay model for a given test agent with references to the host resistance assay panel currently used in our laboratories to screen for alterations in host immunity. Data representative of several kinds of toxicants administered by various routes are presented. These studies are being supported by the U.S. Environmental Protection Agency and the National Institute of Environmental Health Sciences.

METHODS

In studies conducted at IITRI, male and female CD-1 or female B6C3F1 mice were obtained commercially and used when 5 to 8 weeks old. Animals were housed 5 to 10 per cage and received laboratory chow and water ad libitum.

DES ADMINISTRATION

Varying concentrations of the synthetic estrogen DES were injected subcutaneously (sc) in 0.05 ml of corn oil for 14 consecutive days. After 2 to 3 days rest, mice were challenged with an infectious agent or with tumor cells. Details of exposure to other toxicants are appropriately referenced.

HOST RESISTANCE ASSAY PANEL

INFECTIOUS AGENTS

- Listeria monocytogenes was prepared as frozen (-70°C) stock cultures. A modification of techniques described by Dean et al. (1980) and Newborg and North (1980) was used. A cell concentration

of approximately 3 x 10^6 colony forming units (CFU) was used to result in a lethal dose of 10 to 30% (LD_{10-30}) in control mice. Animals were monitored daily for 10 days for death.

- Group C Streptococcus sp., obtained from EPA, was prepared as described by Aranyi et al. (1981a). For intraperitoneal (ip) challenge, approximately 2 x 10^5 CFU of the streptococcal suspension provided an LD_{10-30}. The mice were observed for deaths over a 10-day period. For respiratory challenge, mice exposed to the toxicant and control mice were treated simultaneously with a bacterial aerosol that was generated with a model 841 DeVilbiss nebulizer in a Plexiglas chamber. The 432-liter main compartment is suitable for exposure of 180 mice confined in individual wire cages. Details were described by Ehrlich et al. (1977) and Aranyi et al. (1981a). Mice were observed for 14 days for mortality.

- Influenza A_2/Taiwan/64 virus aerosol challenge procedures were described by Fenters et al. (1979). Animals were observed for 14 days after infectious challenge. Survivors were killed and the extent of pulmonary lesions was expressed as a percentage of total lung consolidation (Horsfall, 1939).

- Herpes simplex virus (HSV) challenge was performed using both Type 1 (S-148 Nahmias strain) and Type 2 (ATCC VR 540). The HSV 1 virus, obtained as a mouse-adapted strain from Schering Corporation, was passaged two times through rabbit kidney cells. The HSV 2 virus was passed once through L929 mouse cells and two times through rabbit kidney cells. In each case, the supernatants were harvested at 3 to 4+ cytopathogenicity (CPE), aliquoted, and frozen away at -70°C. For use, the virus was diluted in phosphate buffered saline (PBS) and an LD_{20-30} dose in 0.2 ml was injected ip.

- Trichinella spiralis infection was performed using modified methods of Larsh and Kent (1949) and Weatherly (1970). Mice were infected by gavage with 0.2 ml aliquots containing approximately 200 larvae. Groups of infected, non-treated control mice were included in all experiments and were killed at 7 days post-infection for adult worm counts (viability control). Experimental and control mice were sacrificed 14 days after infection, the number of adult worms remaining in the small intestine was enumerated microscopically, and the average of three consecutive counts from each mouse was determined.

TUMOR SUSCEPTIBILITY ASSAY

- The B16-F10 melanoma tumor cell line of Fidler (1973; 1975) was obtained from the NCI-Tumor Bank and the procedures followed were essentially the same as he described. Cells were harvested from cultures in the exponential growth phase, pooled, and counted

on a Coulter counter. The cell suspension was adjusted to 2.5 x 10^5 cells/ml with HBSS and 0.2 ml was injected intravenously (iv) into mice within 15 minutes of cell preparation. Mice were sacrificed 3 weeks later, and the lungs were placed into cold water and weighed individually on an analytical balance.

STATISTICAL ANALYSES

At IITRI, three methods were employed for statistical analysis. The mixed-model analysis of variance (ANOVA) as described by Winer (1975) was chosen for continuous response data. In addition to the univariate analyses, multivariate test statistics (Anderson, 1958; Bock, 1975) also were included when there was theoretical justification to believe that simultaneous responses in several biological systems were interrelated. A log-linear model, appropriate for discrete or qualitative response data, was used to evaluate mortality data (Bishop et al., 1975; Haberman, 1979). For between group comparison of survival time, the product limit estimator (Kaplan and Meier, 1958) was used in conjunction with the Mantel-Cox test statistic (Cox, 1972).

RESULTS AND DISCUSSION

• Listeria monocytogenes

This assay system exemplifies a model that may be utilized to detect either increased or decreased host resistance. Listeriosis is regarded as a zoonosis, with a wide range of clinical manifestations ranging from meningitis to urethritis in humans. The pathogenesis is well documented and Newborg and North (1980) have shown that the immune response to Listeria infection is regulated primarily by immunocompetent T-cells and macrophages. This is noteworthy in that some bacterial models evoke primary mechanisms of resistance which are non-specific, e.g., polymorphonuclear leukocyte phagocytosis.

The two parameters measured in this assay were mortality and survival time. Replicate experiments with DES are shown in Table 3. The results show that mortality was increased significantly and mean survival time was decreased in all DES-treated groups relative to controls. These data are similar to those reported by Dean et al. (1980). Note that DES in excess of 0.2 mg/kg was associated with decreasing mortality. Statistical analysis showed that there was a significant quadratic dose-response relationship and the effect was due to a decrease in mortality in the high dosage group in both replicate experiments. This decrease in mortality associated with increasing concentrations of DES may reflect macrophage activation. Boorman et al. (1980) have shown that DES exposure increases both macrophage numbers and activation.

TABLE 3. EFFECT OF DES ON RESISTANCE OF FEMALE B6C3F1 MICE TO LISTERIA MONOCYTOGENES CHALLENGE

DES mg/kg	Mortality D/T[b]	Mortality %	MST[a] (Days)	Number of Replicate Experiments
0	4/20	20	8.8	2
0.2	19/20	95[c]	4.3[c]	
1.0	18/20	90[c]	4.7[c]	
4.0	15/20	75[c]	5.6[d,e]	

[a] Mean survival time
[b] Deaths per total animals
[c] Difference from control: $p \leq 0.0001$
Quadratic dose-response relationship: $p < 0.0001$
[d] Difference from control: $p \leq 0.001$
[e] Difference from 0.2 mg/kg: $p \leq 0.06$

In another study (Table 4), replicate experiments were conducted with cadmium chloride ($CdCl_2$). Mice received 14 daily intragastric (ig) injections of sterile pyrogen-free water containing the $CdCl_2$. In contrast to the DES data, no effects were noted. However, the control mortality (78%) was high and it would be difficult to detect an increase in susceptibility to infection. On the other hand, high control values are required to discern immunopotentiation. In a preliminary study with a maleic anhydride-vinyl copolymer (MVE-2), the agent was given iv at 20 µg/day for 5 days before challenge with Listeria. As shown in Table 5, host resistance was enhanced. The control mortality was 90% and mean survival time was 3.0 days. The experimental group had a 30% mortality and a survival time of 7.6 days. A replicate study has been completed and similar data were obtained.

TABLE 4. EFFECT OF $CdCl_2$ ON RESISTANCE OF FEMALE B6C3F1 MICE TO LISTERIA MONOCYTOGENES CHALLENGE

$CdCl_2$ mg/kg	Mortality D/T	Mortality %	MST[a] (Days)	Number of Replicate Experiments
0	43/55	78	4.8	3
0.4	31/37	84	4.6	
2.0	48/53	91	3.7	
4.0	48/54	89	4.2	
8.0	12/18	67	5.4	

[a] Mean survival time

TABLE 5. IMMUNOSTIMULANT EFFECT OF MVE-2 IN LISTERIA MONOCYTOGENES CHALLENGED FEMALE CD-1 MICE

MVE-2 µg/mouse	Mortality D/T	Mortality %	MST[a] (Days)
0	(9/10)	90	3.0
100	(3/10)	30	7.6

[a] Mean survival time

Thus the Listeria model shows intra- and interlaboratory reproducibility, requires few animals, and is capable of reflecting immunopotentiation as well as immunosuppression.

- **Streptococcus**

This model stresses the importance of the route of administration of both test and challenge agents. Specific immunity against streptococcal infections is not well defined. However, there is evidence suggesting that specific antibody as well as alveolar macrophages and other non-specific phagocytic cells are important in host protection.

INTRAPERITONEAL CHALLENGE

The DES-treated mice were challenged ip with streptococcus and observed for 10 days for mortality and survival time (Table 6). No significant differences in mortality or in survival time between control and DES-treated mice were detected. In another study (Table 7) 80 mg/kg of cyclophosphamide (CY), a well known immunosuppressant, was injected ip either 1 day prior to, or the same day as, ip challenge with streptococcus. Again, there was no difference in mortality when compared to controls.

TABLE 6. EFFECT OF DES ON SUSCEPTIBILITY OF B6C3F1 FEMALE MICE TO STREPTOCOCCUS sp. INTRAPERITONEAL CHALLENGE

DES mg/kg	Mortality D/T	Mortality %	MST[a] (Days)
0	2/21	10	9.5
0.2	3/19	16	9.3
1.0	1/20	5	9.8
4.0	4/18	22	9.1

[a] Mean survival time

TABLE 7. EFFECT OF CY ON SUSCEPTIBILITY OF B6C3F1 FEMALE MICE TO STREPTOCOCCUS sp. INTRAPERITONEAL CHALLENGE

CY mg/kg	Mortality D/T	Mortality %	MST[a] (Days)
0	5/20	25	9.0
80[b]	4/20	20	9.3
80[c]	3/20	15	9.4

[a] Mean survival time
[b] 1 day prior to challenge with streptococcus
[c] 4 hours prior to challenge with streptococcus

AEROSOL CHALLENGE

The results of three replicate experiments in which mice were treated with DES for 14 days, challenged with airborne streptococcus, and observed for 14 days are summarized in Table 8. Analysis of the results showed no significant effect of DES treatment on mortality. However, when mice were injected with 8 mg/kg DES on Days 1 through 5 and 8 through 12, a significant increase in mortality occurred in the DES-treated mice compared with controls. In addition, survival times were found to be significantly decreased following the high dose of DES.

TABLE 8. EFFECT OF DES ON SUSCEPTIBILITY OF B6C3F1 FEMALE MICE TO STREPTOCOCCUS sp. AEROSOL CHALLENGE

DES mg/kg	Mortality D/T	Mortality %	MST[a] (Days)	Number of Replicate Experiments
0[b]	15/68	22.1	12.1	3
0.2	13/73	17.8	12.6	
1.0	20/71	28.2	11.5	
4.0	21/68	30.9	11.6	
0[c]	12/67	17.9	12.3	3
8.0	29/69	42.0[d]	10.9[d]	

[a] Mean survival time
[b] sc injections for 14 days
[c] sc injections Days 1 through 5 and 8 through 12
[d] $p < 0.01$ (Mortality: log linear model; MST: Mantel-Cox test)

When the DES and CY were administered systemically, no changes were found in susceptibility to the respiratory agent, streptococcus, except at one high DES concentration. However, if the route of exposure to a toxicant is inhalation, this host resistance model has been shown to be quite sensitive. In studies reported by Ehrlich et

al. (1978) the relative toxicity of zinc sulfate and ammonium sulfate was assessed using this streptococcal model. Mice inhaled varying concentrations of the sulfates for 3 hours and immediately thereafter were challenged with airborne streptococcus. The data in Table 9 show that inhalation of 1.2 mg/m^3 of zinc sulfate resulted in significant increases in mortality and reduced survival time. In contrast, inhalation of ammonium sulfate, which has been shown to be relatively non-toxic in other assays (unpublished data), had little or no effect on this respiratory infection in concentrations up to 5.3 mg/m^3. The large number of mice required for the aerosol studies should be noted.

TABLE 9. MORTALITY AND SURVIVAL RATE OF CD-1 FEMALE MICE EXPOSED FOR 3 HOURS TO SULFATES AND CHALLENGED WITH STREPTOCOCCUS AEROSOL

SO_4 mg/m^3	$ZnSO_4$ Mortality D/T	$ZnSO_4$ Mortality %	$ZnSO_4$ MST[a] (Days)	$(NH_4)_2SO_4$ Mortality D/T	$(NH_4)_2SO_4$ Mortality %	$(NH_4)_2SO_4$ MST[a] (Days)
0	373/1689	22.1	12.1	233/588	39.6	10.1
$\leq$1.1	125/599	20.9	12.2	22/48	45.8	10.1
1.2-2.0	369/813	45.4[b]	9.1[b]	76/191	39.8	10.7
2.1-3.0	186/278	66.9[b]	6.2[b]	52/96	54.2[b]	9.2
3.1-4.0	---	---	---	47/144	32.6	11.2
$\geq$4.1	---	---	---	46/110	41.8	10.3

[a] Mean survival time

[b] Significant difference from control determined by Chi-square test (mortality) or Student's t test (MST): $p \leq 0.05$.

Data from Aranyi et al. (1981a) further illustrate the usefulness of the streptococcal model in differentiating degrees of toxicity (Table 10). Multiple exposure of mice to 2 mg/m^3 of copper smelter dust resulted in greatly increased mortality whereas no effects were seen in mice exposed to coal fly ash. These investigators also found significantly depressed pulmonary bactericidal activity in mice exposed to the copper smelter dust, whereas no changes in bactericidal activity were found in mice exposed to the fly ash.

Similar host resistance studies with airborne streptococcus were conducted in our laboratory and at the EPA. Exposure to various inhalation hazards such as nitrogen dioxide and/or ozone (Ehrlich et al., 1977; Ehrlich, 1979 and 1980; Gardner et al., 1977a; Gardner, 1980), or aerosols such as sulfate salts (Ehrlich, 1980; Ehrlich et al., 1978), sulfuric acid (Gardner et al., 1977a), cadmium chloride (Gardner et al., 1977b), and arsenic trioxide (Aranyi et al., 1981b), also demonstrated the sensitivity of this assay to inhaled toxicants.

TABLE 10. EFFECTS OF MULTIPLE DAILY 3-HOUR EXPOSURES TO AEROSOLS OF COPPER SMELTER DUST OR COAL FLY ASH ON SUSCEPTIBILITY TO RESPIRATORY STREPTOCOCCUS INFECTION IN CD-1 FEMALE MICE

Aerosol Exposure				Streptococcus Infection		
	Conc., $\mu g/m^3$		No. of Exposures,		%	MST[a]
Sample	Mean	S.D.	3 hr/day	n[b]	Mortality	(Days)
Copper Smelter Dust	2050	190	5	166	33.6***	-2.5***
			10	216	27.2***	-2.9***
			20	268	19.0***	-1.3**
Coal Fly Ash No. 2 (Fluidized-bed)	2040	370	5	325	0.1	-0.2
			10	276	-3.4	0.1
			20	299	1.6	-0.1

[a] Mean survival time
[b] Number of mice
Significant change from corresponding control mice:
** $p \leq 0.05$ *** $p \leq 0.001$

- **Influenza virus** is the second inhalation model demonstrating an effect of route of administration. Humoral immunity and interferon production are the main defenses against influenza infection. Studies (Table 11) with DES, administered by the systemic route, showed no effect on the host's resistance to airborne influenza virus challenge as measured by mortality, survival time, or pulmonary consolidation scores.

TABLE 11. EFFECT OF DES ON SUSCEPTIBILITY OF B6C3F1 FEMALE MICE TO INFLUENZA A_2/TAIWAN/64 AEROSOL CHALLENGE

DES	Mortality		MST[a] (Days)		Pulmonary Consolidation		Number of Replicate
mg/kg	D/T	%	Mean	± S.E.	Mean	± S.E.	Experiments
0	18/47	38.3	12.77	0.77	3.65	0.16	2
0.2	13/48	27.1	13.21	0.70	3.81	0.17	
1.0	18/48	37.5	12.67	0.91	3.65	0.15	
4.0	15/48	31.3	12.87	0.67	3.77	0.18	

[a] Mean survival time

However, as with our streptococcal model, when the toxic test material also was delivered by the inhalation route, an effect was noted with the inhaled respiratory virus. In previous studies in our laboratories (Fenters et al., 1979) male CD-1 mice were exposed 3 hr/day, 5 days/week for up to 20 weeks to respirable-size carbon particles (1.5 mg/m^3) with sulfuric acid (1.4 mg/m^3) adsorbed on

their surface. Data in Table 12 show no effects on mice exposed for 4 weeks. After 20 weeks, however, changes could be detected in mice exposed to acid-coated carbon compared to air or carbon controls. Again, this test system using airborne infectious challenge required a large number of mice.

TABLE 12. LONG-TERM EXPOSURE TO ACID MISTS: RESPONSE OF CD-1 MALE MICE CHALLENGED WITH INFLUENZA A_2/TAIWAN VIRUS

Duration of Exposure (wk)	Experimental Condition	Mortality[a] D/T	Mortality[a] %	MST[b] (Days)	Pulmonary Consolidation
4	Air	16/164	10	13/5	2.05
	Carbon	10/164	6	13.7	1.62
	Acid/Carbon	13/166	8	13.7	1.70
20	Air	48/133	36	12.2	3.11
	Carbon	46/137	34	12.3	3.07
	Acid/Carbon	61/136	45*	11.7**	3.43**

[a] Chi-square test
[b] Mean survival time: Student's t test
* Significant difference ($p \leq 0.10$) compared to carbon control
** Significant difference ($p \leq 0.10$) compared to both air control and carbon

In summary, our studies of airborne bacterial and viral infections have emphasized the importance of the route of administration of the test material. However, they also show that the requirement for fairly large numbers of animals and for costly sophisticated laboratory equipment and engineering controls often obviate the utilization of these models in many laboratories. On the other hand, these host resistance assays are highly sensitive and correlate well with other parameters, such as the pulmonary bactericidal activity, when measuring effects of airborne toxicants. Also, these assays are reproducible between laboratories as attested to by results obtained at IITRI and EPA using the streptococcal model. However, the variation within replicate aerosol assays often is greater than when the infectious challenge is by the systemic route.

- **Herpes simplex virus (HSV)** is another useful model system which has systemic tropism. Cellular immunity, macrophages, and interferon are involved in resistance to herpes virus and it is well documented that members of the herpes virus group cause severe illness in immunocompromised people. Kern (1982) reports that this viral model has been used for detecting immunosuppression as well as immunopotentiation. We have used the herpes mouse model for more than 10 years in our antiviral testing program and we, too, find immunostimulation with compounds from industrial clients. We have used both HSV 1 and 2 since it is not clearly understood if the immune response to these two "different" types is similar.

In our system mice are inoculated ip, allowing the virus to first be detected in the gut, spleen, and liver and ultimately in lung, brain, and spinal cord. Thus there is a generalized infection. In studies with DES (Table 13) there are significant increases in mortality and decreases in survival time between control and DES-treated mice that were infected with HSV 1. Similar increases in mortality were reported by Kern (1982) in mice treated with CY and infected with HSV 1. Data in Table 14, representing HSV 2, are similar. In both cases, the DES significantly suppressed host resistance.

TABLE 13. EFFECT OF DES ON SUSCEPTIBILITY OF B6C3F1 FEMALE MICE TO INFECTION WITH HERPES SIMPLEX VIRUS, TYPE 1

DES mg/kg	Mortality D/T	Mortality %	MST[a] (Days)	Number of Replicate Experiments
0	5/48	10.4	19.8	2
0.2	14/49	28.6[b]	17.2[b]	
1.0	17/48	35.4[d]	16.7[c]	
4.0	16/44	36.4[d]	16.5[c]	

Difference from control determined by Mantel-Cox test (MST) or by log linear analysis (mortality):

[a] Mean survival time
[b] $p < 0.05$
[c] $p < 0.01$
[d] $p < 0.001$

TABLE 14. EFFECT OF DES ON SUSCEPTIBILITY OF B6C3F1 FEMALE MICE TO INFECTION WITH HERPES SIMPLEX VIRUS, TYPE 2

DES mg/kg	Mortality D/T	Mortality %	MST[a] (Days)	Number of Replicate Experiments
0	8/70	11.4	19.8	4
0.2	12/69	17.4	19.1	
1.0	17/62	27.4[b]	17.1[c]	
4.0	19/66	28.8[b]	17.7[c]	

Difference from control determined by Mantel-Cox test (MST) or by log linear analysis (mortality):

[a] Mean survival time
[b] $p < 0.05$
[c] $p < 0.01$

A neglected area of research is the effect of toxic agents on the secondary immune response. We recently initiated studies with cadmium chloride and evaluated both primary and secondary immune responses to HSV 1. As Table 15 shows, with primary virus challenge the control and experimental mortality rates were similar and, as expected, complete protection from death was seen after the secondary challenge. Thus $CdCl_2$ treatment when administered before the primary challenge did not interfere with the development of a strong immune response.

TABLE 15. EFFECT OF $CdCl_2$ ON SUSCEPTIBILITY OF B6C3F1 FEMALE MICE TO PRIMARY AND SECONDARY INFECTION WITH HERPES SIMPLEX VIRUS, TYPE 1

$CdCl_2$ mg/kg	Primary Challenge[a] Mortality D/T	Mortality %	MST[c] (Days)	Secondary Challenge[b] Mortality D/T	Mortality %	MST[c] (Days)
0(1°)	5/20	25.0	17.8	0/15	0***	21.0***
0.4	2/26	7.7	19.9	0/24	0***	21.0***
2.0	4/18	22.2	18.5	0/14	0***	21.0***
4.0	3/18	16.7	18.6	0/15	0***	21.0***
0(2°)	----	----	----	11/20	55.0	14.2

[a] $CdCl_2$ was given ig for 12 days: 3 days later mice were challenged ip with herpes virus

[b] Secondary challenge with virus was administered 21 days after the primary challenge to the surviving mice and to an additional group of distilled water-treated mice which served as control for the second challenge

[c] Mean survival time

Significant difference from control determined by Chi-square test (mortality) or Student's t test (MST):

***$p \leq 0.001$

The questions remain

- what would be the effect on the secondary response when using a known immunosuppressant such as CY or DES before primary challenge?
- what effect would occur on the secondary response when an immunosuppressant or immunopotentiator was given after the primary microbial or tumor challenge? Such studies are in progress in our laboratories.

Thus the herpes model gives reproducible data, requires relatively small numbers of mice, and is capable of evaluating suppression as well as potentiation of the immune response.

• EXPULSION OF TRICHINELLA SPIRALIS

Delayed hypersensitivity and cellular immunity are important in defending a host against T. spiralis (Larsh et al., 1974). Immunity to T. spiralis larvae rids infected mice of the adult parasite through expulsion from the gut in about 14 days. Thus the presence of an increased number of adult parasites at Day 14 indicates impaired host resistance and immunologic changes.

Studies by Dean et al. (1980) showed that a concentration as low as 0.2 mg/kg of DES suppressed the immune system sufficiently to allow significant increases in the number of adult worms. We obtained similar results as shown in Table 16. This assay, as with Listeria, is so sensitive that as few as 10 mice can be used to obtain reproducible data for interlaboratory validation. Unlike Listeria, we have seen no published reports showing the usefulness of this model system to study immunopotentiation.

TABLE 16. EFFECTS FROM DES ON EXPULSION OF ADULT TRICHINELLA SPIRALIS FROM B6C3F1 FEMALE MICE

DES mg/kg	N	Average Number of Adult Worms at Day 14	% Expulsion Relative to Controls at Day 7
0	10	0	100
0.2	10	0	100
1.0	10	2.9[a]	89.3[a]
4.0	10	11.3[a]	58.2[a]

[a] $p \leq 0.0001$ (Mixed model ANOVA)

TUMOR MODELS

As stated earlier, care should be exerted in the selection of a given model dependent upon the agent being tested, the route of exposure to the agent, and the flexibility of the model for measuring stimulatory as well as adverse effects on host resistance. To illustrate this point, we have selected three tumor models (Table 17) for examination: the 4198T tumor line, a polyoma virus-induced tumor line developed by Dr. Robert Ting; the PYB6 sarcoma, a polyoma virus-induced tumor line; and the B16-F10 melanoma tumor line of Dr. I. J. Fidler, a metastatic line developed for the ability to form pulmonary tumor foci.

TABLE 17. CONSIDERATIONS FOR SELECTING TUMOR SUSCEPTIBILITY MODELS

Tumor	Site of Implantation	Useful for Measuring: Decreased Host Resistance	Useful for Measuring: Increased Host Resistance	Endpoint Measurement
4198T	sc	maybe	no	Mortality, tumor incidence or growth rate
PYB6	sc	yes	maybe	Mortality, tumor incidence or growth rate
B16-F10	iv	yes	yes	Number of tumor foci, lung weights, IUDR incorporation

The 4198T tumor cell line requires approximately 10^6 cells/mouse, injected sc, to produce a tumor incidence of only 10 to 20% within 60 days. In addition to requiring this relatively large number of cells and the expense of growing sufficient cells in culture, the model effectively can measure only decreased host resistance since too many cells would be required to produce a 70-80% tumor incidence. In fact, this model has not been found particularly satisfactory even for measuring decreased resistance (Dean, personal communication).

The PYB6 tumor requires only about 5×10^3 cells sc to produce a 10 to 40% incidence and this model has been utilized to demonstrate decreased host resistance from exposure to a number of agents including DES (Dean et al., 1982a). Although it should be useful for detecting increased host resistance, we have seen no reports to that effect as a result of exposure to any test agent.

In contrast, the B16-F10 melanoma cell model, which requires about 5×10^4 cells/mouse, has been shown to be sensitive in detecting decreased host resistance using CY and the phorbol ester TPA (Dean et al., 1982a) as well as increased host resistance (Table 18). In our study, 3 weeks after iv challenge with 5×10^4 B16-F10 melanoma cells, the mice were killed and lungs removed. In two replicate experiments, this concentration of tumor cells resulted in lung tumor foci too numerous to count and a statistically significant reduction of tumor mass in the lungs of all the DES-treated animals as compared with the controls. These data contrast with the findings of Dean et al. (1982a) with DES and the PYB6 model. A possible explanation is that B16 melanoma cells are highly sensitive to activated macrophages (Fidler, 1974) and DES is reported to be a potent macrophage activator (Boorman et al., 1980). Whether this activation is reflected as cytostasis or is cytocidal remains to be determined and indicates further potential usefulness for the B16-

F10 model in helping delineate mechanisms of action of certain agents. Finally, because the lungs are the site of tumor growth, this model should be particularly useful in evaluating effects of inhaled toxicants.

TABLE 18. EFFECT OF DES ON TUMOR GROWTH IN THE LUNGS OF B6C3F1 FEMALE MICE FOLLOWING IV INOCULATION OF 5 X 10^4 B16-F10 MELANOMA CELLS

DES mg/kg	N	Lung Weights (mgs) Mean	± S.D.	Number of Replicate Experiments
0	20	824	476	2
0.2	20	530[a]	427	
1.0	20	553[a]	360	
4.0	19	523[a]	282	

[a] $p \leq 0.004$ (Mixed model ANOVA)

The uses of tumor models in immunotoxicology will be more extensively discussed by Dr. Kerkvliet in the following paper.

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PAPER NO. 5

THE USE OF TUMOR MODELS IN IMMUNOTOXICITY TESTING

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The role of the immune system in the prevention and control of tumor growth has been a rapidly expanding area of research during the past decade. The concept that a host could immunologically recognize and thus eliminate transformed cells from the body offered many new prospects for the prevention and treatment of cancer. From a toxicology standpoint, however, immunologic control of tumor growth presented an additional area for concern - that is, that even if a chemical was not directly carcinogenic, it might, through effects on the immune system, create an individual that was more susceptible to tumor growth, and thus present an even more subtle hazard than a direct carcinogen.

Because of the biologic and toxicologic relevance of a tumor model, our laboratory has been working to develop suitable tumor models for use in immunotoxicity testing. In addition to the toxicologic significance of altered tumor susceptibility due to chemical exposure, we felt that a tumor model could also provide several testing advantages from a technical standpoint. The first major advantage of a tumor model is the availability of multiple in vivo endpoints for assessing altered host susceptibility. These include tumor frequency, tumor latency and growth rate, progression versus regression, and metastases development. The availability of more than a single all-or-nothing endpoint such as mortality should provide increased sensitivity for detecting immunologic alterations due to chemical exposure.

The second major advantage of a tumor model is the ability to quantitate essentially all major aspects of the immune response within a single system. These include the surveillance mechanisms of natural killer (NK) cells and macrophages which control susceptibility to tumor growth as well as specific antitumor immune responses which control tumor growth rate and perhaps metastases development. Specific antitumor host defenses include cytolytic T cells, cytolytic and cytostatic macrophages, complement-dependent cytotoxic antibodies, antibody-dependent K cell cytotoxicity, and

lymphokine production. Furthermore, in a tumor model one can also examine the immunologic mechanisms whereby tumors can escape from antitumor defenses, including the level of serum blocking factors and suppressor cell activity.

The third major advantage of a tumor model is that all the responses are quantitated in terms of a common antigenic stimulus. Using the tumor cells as antigen provides a common basis by which all the in vitro functional assays can be compared and, more important, correlated to the phenomenon of in vivo tumor growth.

Several tumor systems have been used in immunotoxicity assessments (Table 1). The majority are mouse tumors and their use has been limited to the study of in vivo host susceptibility. Both virus and chemically induced tumors, as well as spontaneous tumor models have been utilized. However, a lack of interlaboratory comparison of sensitivities makes it difficult to recommend one model over another. Advantages of each model may depend on the experimental conditions. For example, lung tumor models may be of greatest relevance in inhalation exposure studies. The ability of the tumor to metastasize may also be a relevant factor in some studies.

TABLE 1. TUMOR MODELS USED IN IMMUNOTOXICITY ASSESSMENT

Tumor	Strain of Origin	Endpoints	Reference
MKSA (SV-40 virus)	BALB/c	Tumor frequency, latency, volume	Dean et al., 1979
PYB6 (polyoma virus)	C57BL/6	Tumor frequency, latency, volume	Dean et al., 1980
MSV-MSB (Moloney sarcoma virus)	C57BL/6	Tumor frequency, progression, secondary challenge	Kerkvliet et al., 1979, 1980, 1982a,b
RLV (Rauscher leukemia virus)	C57BL/6	Leukemia and death	Gainer, 1972
MOPC-104	BALB/c	Time to death	Bellanti et al., 1978
Walker carcinosarcoma	Sprague-Dawley	Tumor frequency, latency, volume, metastases	Kerkvliet and Kimeldorf, 1977
Lewis lung tumor	BALB/c	Lung tumor nodules	Dean et al., 1980
B16F10 melanoma	C57BL/6	Lung tumor nodules	Dean et al., 1982

Three mouse tumor models have been primarily utilized in our laboratory for the study of chemical-induced immunotoxicity. Their sensitivity in detecting immune alterations induced by exposure to pentachlorophenol (PCP), a widely used wood preservative, will be described (Kerkvliet et al., 1982a). In these studies, C57BL/6 female mice, 8 weeks of age, were placed on diets contaminated with 50 or 500 ppm pure (99+%) or technical (86%) grade PCP. The diets

were available to the animals ad libitum for 8 weeks prior to tumor challenge. The commercially available technical PCP is known to be contaminated with significant levels of other chlorinated phenols as well as chlorinated dibenzofurans and dioxins.

The first tumor model assesses the ability of the animals to resist challenge with a syngeneic tumor given at a low tumor dose, producing a 10-30% incidence of progressive tumors in normal, untreated mice. Low dose tumor challenge is considered a sensitive assessment of general immunocompetence, with tumor resistance likely representing the surveillance mechanisms of NK cells and/or macrophages. We used a methylcholanthrene-induced sarcoma of C57BL/6 mice injected subcutaneously at a dose of 1×10^4 tumor cells. Mice were examined three times per week for appearance of tumors, and, once palpable, tumor growth rates were estimated by caliper measurements of the tumor diameters.

Exposure of mice to technical grade PCP resulted in a significant increase in susceptibility to low dose tumor challenge (Table 2). The incidence of progressive tumor growth increased from 35% in control mice to 67 and 82% in animals exposed to 50 and 500 ppm technical PCP, respectively. Animals exposed to pure PCP at the same dietary levels did not show any significant alteration in tumor susceptibility with a tumor incidence of 31 and 40% in the 50 and 500 ppm exposure groups, respectively. The effect of technical PCP was observed only at the level of initial susceptibility; the growth rates of the tumors that developed and host survival time were not significantly different from controls.

TABLE 2. EFFECT OF PCP EXPOSURE ON SUSCEPTIBILITY OF MICE TO LOW-DOSE TUMOR CHALLENGE[a]

Treatment ppm PCP	Progressive Tumor Incidence[b] (%)
0	9/26 (35)
50 pure	4/13 (31)
500 pure	6/15 (40)
Dose Response (P)	NS
50 technical	10/15 (67)
500 technical	9/11 (82)[c]
Dose Response (P)	<0.005

[a] Mice were injected with 1×10^4 Sarcoma 1412 cells s.c. and were observed twice weekly for 90 days.

[b] Number of mice with tumor/number of mice injected.

[c] Significantly different from 0 ppm, χ^2, $p < 0.05$.

The second tumor model that we have utilized most extensively is the Moloney sarcoma virus (MSV)-induced tumor system in C57BL/6 mice. The MSV system is an attractive model for immunotoxicity assessments because of the predictable and short induction time of strongly antigenic tumors which leads to spontaneous tumor regression in immunocompetent hosts (Levy and Leclerc, 1977). Tumors induced by MSV appear in 5-10 days at the site of virus injection, reach a peak size around day 14, and completely regress by day 21. The mechanisms of tumor regression have been well-studied, with tumor regression primarily dependent on an intact cytotoxic T lymphocyte response. However, growth inhibitory macrophages (Holden et al., 1976; Korn et al., 1978a,b), cytotoxic antibodies (Leclerc et al., 1972; Lamon et al., 1973), and antibody-dependent K cell cytotoxicity (Pollack, 1973; Pollack et al., 1972) also appear to play a role in MSV tumor regression. Furthermore, animals that have undergone primary MSV tumor growth and regression retain specific antitumor immunity (Holden et al., 1975), rendering the animals resistant to a secondary challenge with MSV-transformed tumor cells (MSB) injected at a dose that produces a 100% incidence of progressive tumors in non-MSV immune animals. Thus, the MSV model is useful for the examination of toxicant effects on both primary and secondary antitumor immune responses.

The applicability of the MSV system to secondary challenge has proven to be a very useful aspect of the model as it appears to provide increased sensitivity in detecting immunosuppression induced by chemical exposure. In studies to further assess the immune suppression induced by PCP exposure, mice were exposed to diets contaminated with 50 or 500 ppm pure or technical PCP. After 8 weeks of exposure, the mice were inoculated intramuscularly in the left hind leg with MSV. Primary tumor growth and regression was monitored daily, and, after 2 months, all regressor animals were reinoculated in the right hind leg with 1×10^6 MSB tumor cells. The animals were monitored for an additional 2 months for appearance of MSB tumors, after which time all survivors were killed and necropsied.

Results of this study are summarized in Table 3. Following MSV injection, all control and PCP-exposed animals developed primary tumors at the site of virus inoculation with peak tumor size observed on days 9-10 followed by tumor regression. However, on approximately day 18, 55% of the animals exposed to 500 ppm technical PCP exhibited a recurrence of tumor growth which progressed until the death of the host. Complete tumor regression occurred in all other groups. Following secondary challenge with MSB, the incidence of progressive tumor growth was significantly elevated in animals exposed to 50 ppm technical PCP; two of five animals exposed to 500 ppm technical PCP that survived the initial MSV challenge also developed progressive MSB tumors. The combined mortality from primary and secondary tumor challenges was thus increased from 19% in controls to 45 and 73% in animals exposed to 50 and 500 ppm

technical PCP, respectively. As with the low-dose tumor challenge model, animals exposed to pure PCP did not show enhanced MSV-MSB tumor susceptibility.

TABLE 3. EFFECT OF PCP EXPOSURE ON SUSCEPTIBILITY OF MICE TO PROGRESSIVE PRIMARY MSV-INDUCED TUMOR GROWTH AND RESISTANCE TO SECONDARY MSB CHALLENGE[a]

Treatment ppm PCP	Progressive Tumor Incidence (%)		
	Primary MSV	Secondary MSB[b]	Total MSV/MSB
0	0/16 (0)	3/16 (19)	3/16 (19)
50 pure	0/10 (0)	1/10 (10)	1/10 (10)
500 pure	0/11 (0)	2/11 (18)	2/11 (18)
Dose Response (P)			NS
50 technical	0/11 (0)	5/11 (45)	5/11 (45)
500 technical	6/11 (55)[c]	2/5 (40)	8/11 (87)[c]
Dose Response (P)			<0.005

[a] Mice were injected with MSV in the right hind leg; primary tumor growth and regression/progression were monitored for 105 days. Regressor animals were reinoculated in the left hind leg with 1 x 10^6 MSB cells. Secondary tumor growth was monitored for an additional 50 days.

[b] Six of six non-MSV immunized mice inoculated with 1 x 10^6 MSB cells died from progressive tumor growth.

[c] Significantly different from 0 ppm, χ^2, $p < 0.05$.

However, when animals that were resistant to both MSV and MSB challenges were necropsied, sarcoma development was unexpectedly observed in the spleen of several PCP-exposed animals. The incidence of splenic tumors was 50% (3/6) in animals exposed to 50 ppm technical PCP, 22% (2/9) in animals exposed to 50 ppm pure PCP, and 44% (4/9) in animals exposed to 500 ppm pure PCP. Splenic tumor formation was not observed in any of the 13 surviving control animals nor in the 3 animals remaining in the 500 ppm technical PCP group. The development of splenic tumors following MSV injection is a phenomenon that we have not previously observed in normal animals during our 4 years of work with the MSV system. However, metastases of MSV to the spleen has been reported to occur in immunoincompetent newborn mice (Perk and Moloney, 1966) and in adult mice immunosuppressed by x-irradiation or neonatal thymectomy (Stanton et al., 1968). These results suggest that splenic tumor development may provide a highly sensitive parameter for detecting immune suppression induced by exposure to environmental chemicals.

The third tumor model that has been used in our laboratory for assessing chemical-induced immunotoxicity is an allograft model, the DBA/2 ($H\text{-}2^d$) P815 mastocytoma transplanted into C57BL/6 ($H\text{-}2^d$) mice. This model has been used primarily for the in vitro assessment of tumor immunity. Allogeneic sensitization of test animals by a single intraperitoneal injection of tumor cells results in a strong immune reaction directed against the histocompatibility antigens on the tumor cells. This immune response is expressed in high levels of cytotoxic T cell activity, high cytotoxic antibody titers, and activation of the mononuclear phagocyte system. All these responses can be readily measured in a single animal, using spleen cells for T cell cytotoxic activity, serum for antibody titration, and peritoneal exudate cells for macrophage activation. In addition, under circumstances of severe immunodepression, the in vivo growth of the allogeneic tumor can be used as a host susceptibility assay. The P815 tumor model also offers several advantages from a technical standpoint since quantitation of all the in vitro assays is based on the efficient and objective measurement of radioactive label release or uptake. T cell cytotoxicity, for example, is easily measured by the in vitro lysis of ^{51}chromium-labelled tumor cells (Brunner et al., 1970). The methodology involves the labelling of the P815 tumor cells with ^{51}Cr followed by a 4-hour incubation of the labelled tumor cells with serial dilutions of spleen cells that were obtained from animals injected 10 days previously with 1×10^7 P815 tumor cells. During incubation the cytotoxic T cells present in the spleen cell suspension lyse the tumor cells causing the release of ^{51}Cr into the medium. Following centrifugation, an aliquot of the cell-free supernatant is harvested and the amount of radioactivity present is quantitated on a gamma scintillation counter. The amount of ^{51}Cr released is then plotted against the ratio of spleen cells to tumor cells assayed. Cytotoxic activity can then be expressed in terms of lytic units (LU) where 1 LU is equal to the number of spleen cells causing 50% lysis of the tumor cells. Based on the number of spleen cells recovered per animal, the number of LU per spleen can be calculated. This same ^{51}Cr release assay can be used to quantitate antibody and macrophage cytotoxicity, simply by substituting serially diluted serum (in the presence of complement) or peritoneal cells for the spleen cells.

Thus far we have only utilized the cytotoxic T cell assay in immunotoxicity studies of PCP, with the assays for cytotoxic antibody and macrophages under development. As shown in Figure 1, animals exposed to technical PCP contaminated diets exhibited an exposure level-dependent reduction in splenic T cell cytotoxicity. The reduction was statistically significant at all effector:target cell ratios in the 500 ppm technical PCP group. No change in lytic activity was apparent using cells from mice exposed to pure PCP. The similarity in the slopes of the curves suggested that there was no qualitative difference in lytic activity between the cytotoxic cells from technical PCP-exposed and control animals, but rather a reduction in the percentage of cytotoxic cells present in the spleen of technical PCP-exposed mice. Thus, as shown in Table 4, on a LU

basis, essentially twice as many spleen cells were required from the 500 ppm technical PCP exposed mice to lyse the same number of tumor cells as control. Coupled with a reduction in the total number of spleen cells recovered from the 500 ppm technical PCP exposed animals, a 55% reduction in the number of LU/spleen was observed. These results directly support the previous findings of enhanced tumor susceptibility observed in vivo in technical PCP-exposed animals, and suggest that the mechanism of enhanced tumor growth may be due to a reduction in T cell cytotoxicity induced by exposure to PCP.

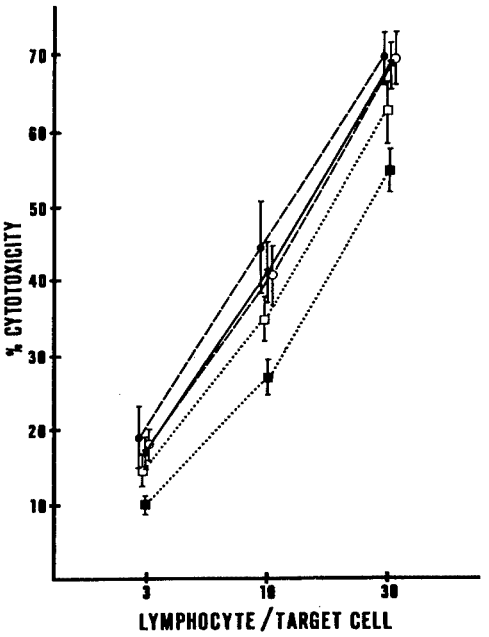

Figure 1. Effect of PCP exposure on T cell-mediated cytotoxic activity of spleen cells from P815-allogeneic sensitized mice. Cytotoxicity determined in a 6 hour ^{51}Cr-release assay using P815 mastocytoma cells as targets. Data represent mean ± SE of the cytotoxic response of 4-6 animals tested individually. X ——— control, o --- 50 ppm pure PCP, ● ---- 500 ppm pure PCP, □ ····· 50 ppm technical PCP, ■ ····· 500 ppm technical PCP.

TABLE 4. EFFECT OF PCP EXPOSURE ON CYTOTOXIC T CELL ACTIVITY[a]

Treatment ppm PCP	LU_{50}[b] (x 10^{-4})	Spleen Cell[c] Recovery (x 10^{-7})	LU/Spleen (% of Control)
0	12.5 ± 1.7	13.5 ± 0.6	11,284 ± 1,264
50 pure	13.0 ± 2.7	11.7 ± 0.8	10,112 ± 1,227 (90)
500 pure	12.4 ± 1.7	12.0 ± 0.5	10,689 ± 1,818 (95)
50 technical	17.3 ± 4.0	14.4 ± 1.4	9,823 ± 2,424 (87)
500 technical	24.0 ± 3.3[d]	10.8 ± 0.8[d]	5,102 ± 1,018[d] (45)

[a] Values presented as mean ± se, 4-5 animals/group.

[b] LU_{50} defined as the number of spleen cells required to lyse 50% of 1 x 10^4 ^{51}Cr-labelled P815 tumor cells in 6 hours.

[c] Spleen cell recovery after 10 sec. hypotonic lysis of RBCs; viability >90% in all groups.

[d] $P < 0.05$, Student's t test.

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SESSION II

IMMUNOTOXICOLOGY SPECIFIC CHEMICALS

Chairman

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PAPER NO. 6

HEAVY METAL MODULATION OF LYMPHOCYTE AND MACROPHAGE ACTIVITY

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INTRODUCTION

To determine the immunotoxic potential of an environmental factor, the experimental protocols should include evaluation of host resistance to extracellular and intracellular parasites which requires intact humoral immunity and cell-mediated immunity, respectively. Assessment of host resistance to an extracellular parasite such as Streptococcus pneumoniae and an intracellular parasite such as Listeria monocytogenes (Figure 1) constitutes a holistic approach because resistance to pneumococci requires T-cell, B-cell, and macrophage interactions for the production of specific antibodies which activate the complement cascade and aid phagocytosis (Wood, 1941; Winkelstein et al., 1972), and resistance to Listeria requires T-cell and macrophage interactions for T-cell production of lymphokines which enhance macrophage bactericidal mechanisms (Schell and Lawrence, 1977; Mackaness, 1970; North, 1973). In addition to the investigation of host resistance, the immune system must be dissected into its functional components and the effects of an environmental agent examined on each separately and combined, so that the mechanism(s) of their differential immunomodulatory potential can be examined. To dissect T-cell and B-cell reactivities, additional assays shown in Figure 1 can be employed. For the development of antibody-producing cells, the ability of an agent to interfere with humoral immunity to a T-dependent and T-independent antigen can be assessed.

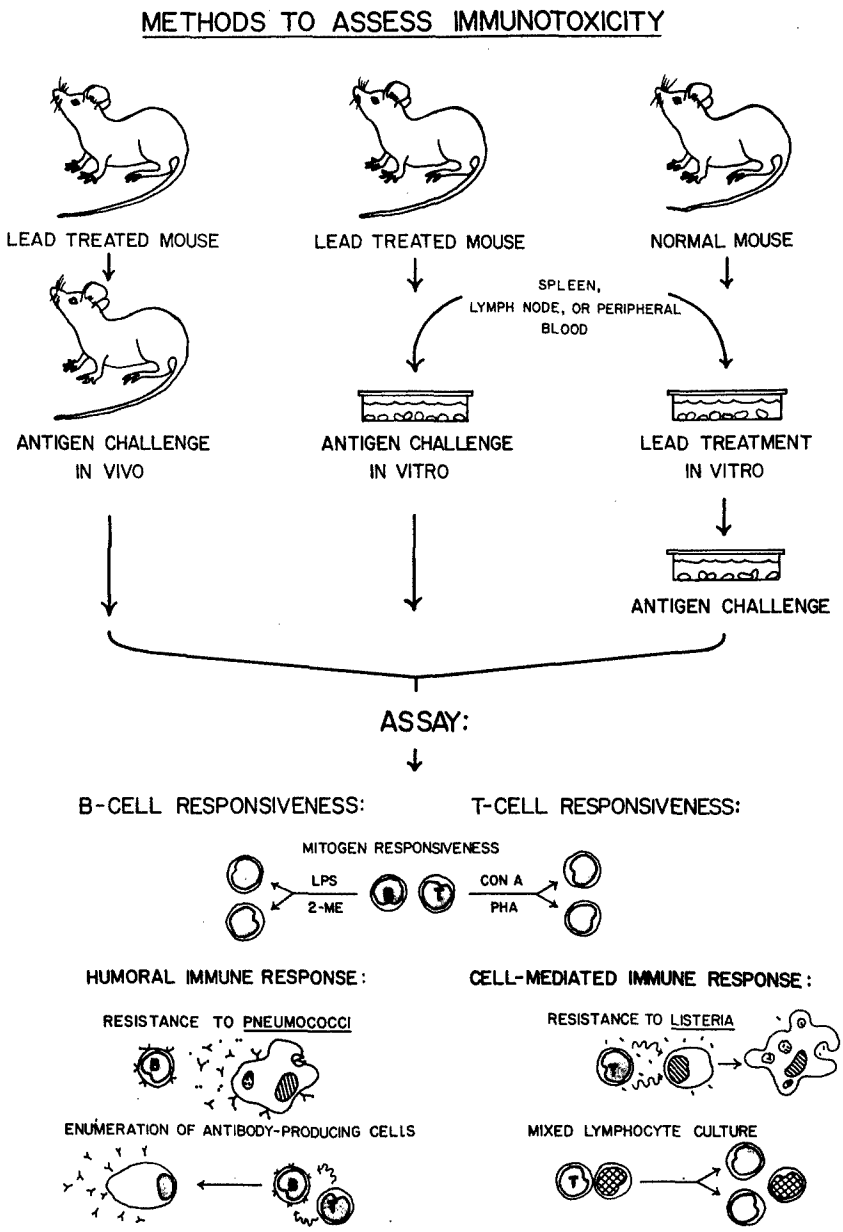


Figure 1. Methodology for the assessment of the immunotoxicity of an environmental factor.

Experimental exposures to an environmental agent should include the natural route of exposure. However, in vitro exposures can be helpful in the assessment of the direct influence of a reagent on a particular cell type. In vivo exposures may influence secondary factors such as cellular trafficking, ontogenic development of cell subsets, and accessory cell functions. For complete analysis, in vivo and in vitro studies should be compared. We have investigated the in vivo and in vitro effects of lead (Pb) on humoral and cell-mediated immunity (Lawrence, 1981a,b,c), and these effects are not always comparable, as will be discussed in this presentation.

Acute and chronic exposure of experimental animals to low levels of Pb have been shown to alter the immune system and enhance

a host's susceptibility to bacterial (Lawrence, 1981c; Cook et al., 1975; Hemphill et al., 1971; Selye et al., 1966) and viral (Gainer, 1974; Gainer, 1977; Vengris and Mare, 1974) infections. Although Pb has been shown to enhance morbidity and mortality in infected animals (Cook et al., 1974; Vos, 1977), the mechanisms involved have not been delineated. In our previous studies (Table 1; Lawrence, 1981a), we have shown that Pb, unlike many other heavy metals, enhanced in vitro primary humoral immune responses to a T-dependent antigen, sheep red blood cells (SRBC). This in vitro immunopotentiation by Pb was unexpected since most earlier reports indicated that in vivo Pb was immunosuppressive (Koller, 1973; Koller and Kovacic, 1974). However, an acute Pb exposure (single intraperitoneal injection of 4 mg) was reported to enhance the in vivo primary humoral immune response to SRBC (Koller et al., 1976). When we orally exposed CBA/J mice to 0.08 to 10 mM Pb for 1 to 10 weeks and immunized these mice with SRBC (Phase I, Figure 1), we were unable to detect a significant expressive or enhancing effect (Figure 2) although the host resistance of these mice to Listeria infection was significantly reduced (Figure 3; Lawrence, 1981c). In addition, we assayed the in vitro reactivity of spleen cells from the in vivo exposed CBA/J mice and discovered that in vivo exposure to Pb (0.08 and 0.4 mM, immunoenhanced; 10 mM, immunosuppressed) did alter in vitro humoral immune responses (Phase II, Figure 1; Lawrence, 1981c). We continue to gather data from immunologic studies designed to investigate the influence of in vivo and in vitro exposure to Pb on humoral immunity and cell-mediated immunity. In this report, we describe the effects of acute in vivo exposure (0.5 mg Pb intravenously) and chronic in vivo exposure (52-56 weeks oral exposure to 0.1 to 10 mM Pb) on in vivo and in vitro immunoassays including macrophage activity, resistance to Listeria, mixed lymphocyte culture (MLC) activity, and plaque-forming cell (PFC) development, as well as renal immunopathology.

TABLE 1. INFLUENCE OF METALS ON THE IN VITRO PRIMARY HUMORAL IMMUNE RESPONSE TO SRBC[a]

Potentiator	No Effect	Inhibitor
Pb^{++}	Ca^{++}	Hg^{++}
Ni^{++}	Fe^{++}	Cu^{++}
		Mn^{++}
		Cd^{++}
		Co^{++}
		Cr^{+++}
		Zn^{++}
		Sn^{++}

[a] The effects of various concentrations (10^{-4} to 10^{-7} M) of metal chlorides were assessed by addition at initiation of culture. After 5 days in vitro, the number of SRBC-specific PFC were enumerated and the ability of a metal to alter the primary humoral immune response determined (Lawrence, 1981a).

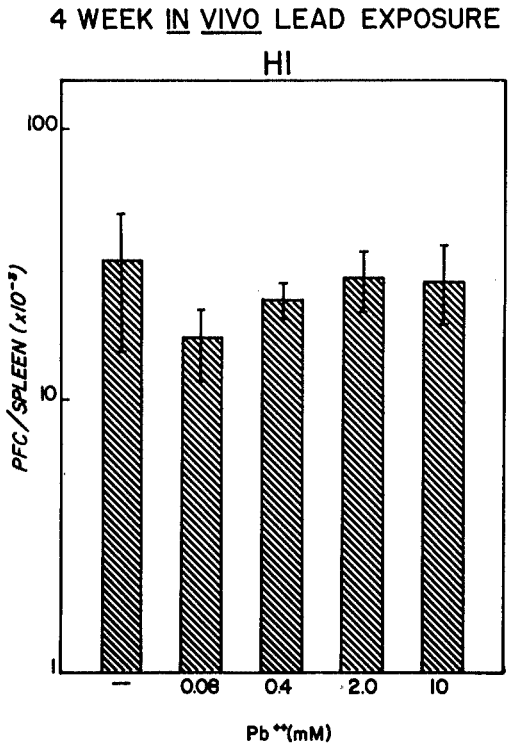


Figure 2. In vivo humoral immunity of CBA/J mice exposed orally to various doses of Pb. The number of SRBC-specific PFC were assessed five days after immunization with 10^8 SRBC. Each bar represents the mean ± S.D. of five mice/group. Taken from reference 8 with permission from Infection and Immunity.

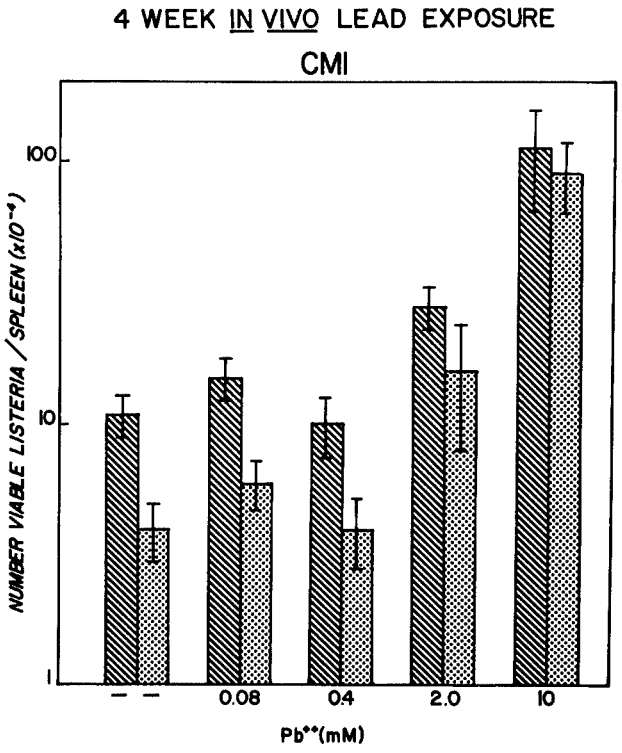


Figure 3. In vivo assessment of the influence of Pb (4 week oral exposure) on host resistance to Listeria. The log number of viable Listeria/spleen were enumerated 48 (hatched bars) and 72 (dotted bars) hours after infection. There was 100% mortality in the 0.4, 2, and 10 mM groups by 7 days post-infection (Day +7). Taken from reference 8 with permission from Infection and Immunity.

RESULTS AND DISCUSSION

As shown in Figures 2 and 3, Pb fed to CBA/J mice for 4 weeks did not significantly alter their humoral immune response to SRBC, but did reduce host resistance to Listeria (Lawrence, 1981c). For comparative purposes, mice were exposed acutely to Pb (intravenous injection of 0.5 mg $PbCl_2$). When the Pb was injected on the same day as immunization with 10^8 SRBC, there was a significant enhancement in the number of PFC (Figure 4). This was our first indication of in vivo immunopotentiation by Pb. On the other hand, this acute exposure to Pb on the day before (Day -1) or day after (Day +1) infection with Listeria increased host susceptibility in that the number of viable Listeria recovered 48 and 72 hours post-infection was increased significantly (Figure 5). The Day +1 and Day -1 groups had 100% mortality; the group given Pb on Day -7 and the control group had >50% survival. Unlike the humoral immune difference, the chronic (4 week daily exposure) and acute effects of Pb on host resistance to Listeria (cell-mediated immunity, CMI) were similar. The acute exposure to Pb given one day before or on the day of removal of spleen cells did reduce in vitro MLC responsiveness (Table 2) more than had been reported for chronic exposure (4 weeks), in which the highest dose (10 mM) only produced a 24% inhibition (Lawrence, 1981c). Interestingly, in vivo Pb exposure and in vitro assessment of humoral immunity (PFC development) was similar for chronic and acute exposure to Pb. As shown in Table 3, injection of 0.5 mg $Pbcl_2$ produced no effect or enhanced the SRBC-specific PFC response in vitro. In our previous study, we reported that after a 4 week oral exposure to 0.08 or 0.4 mM Pb, the in vitro PFC response to SRBC also was enhanced; however, a 10 mM dose was suppressive (Lawrence, 1981c).

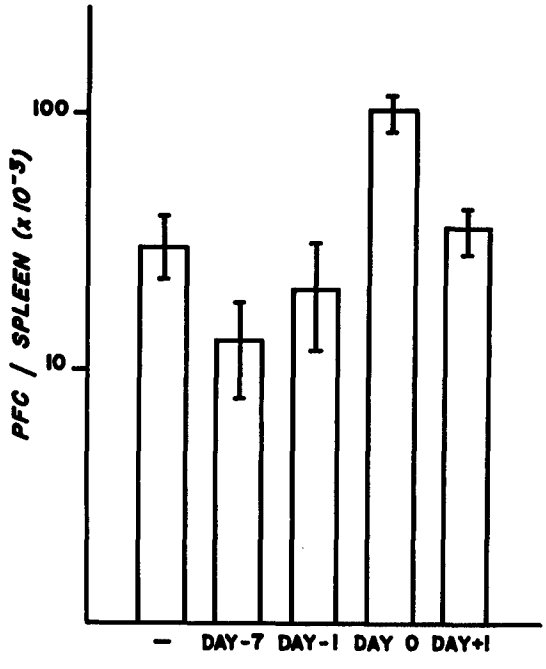


Figure 4. In vivo humoral immunity was assessed after acute exposure to Pb (intravenous injection of 0.5 mg $PbCl_2$). The numbers of SRBC-specific PFC were assessed five days after immunization with 10^8 SRBC (Day 0). Each bar represents the mean ± S.D. of five mice/group.

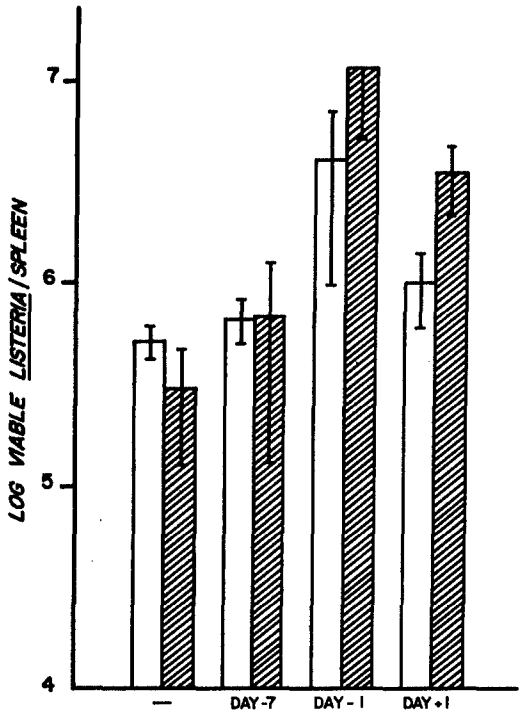


Figure 5. In vivo assessment of the influence of acute Pb exposure (0.5 mg $PbCl_2$ injected intravenously before, with, or after Listeria) on host resistance to Listeria. The log number of viable Listeria/spleen were enumerated 48 (open bars) and 72 (hatched bars) hours after infection. Each bar represents the mean ± S.D. number of viable Listeria/spleen from 5 mice. There was 100% mortality in the Day -1 and Day +1 groups by Day +7.

TABLE 2. EFFECT OF LEAD (0.5 mg IV) ON MLC RESPONSE OF CBA/J MICE TO BDF_1 MICE

Lead Treatment	Stimulator Cells[a]		S.I.[b]	% Control
	CBA/J	BDF_1		
--	19,544[c] ±2,187	153,353 ±15,989	7.8	100
Day -7	25,963 ±1,567	162,130 ±11,352	6.3	81
Day -1	20,705 ±2,343	99,818 ±17,307	4.8	61
Day 0	21,698 ±3,045	105,763 ±13,465	4.9	62

[a] Stimulator cells were the syngeneic CBA/J spleen cells or the allogeneic BDF_1 spleen cells; a ratio of 2 stimulators: 1 responder was used, the stimulator cells were irradiated with 2000 R before addition to the MLC, and the MLC was pulsed with 1 µCi ^3H-thymidine for 24 hr on Day 4.

[b] S.I. = stimulation index (cpm BDF_1:CBA/J/cpm CBA/J:CBA/J).

[c] The number represents the mean from triplicate cultures ± standard deviation.

TABLE 3. EFFECT OF LEAD (0.5 mg IV) ON IN VITRO PRIMARY HUMORAL IMMUNE RESPONSE TO SRBC[a]

Day of Pb Treatment[b]	PFC/Culture	% Control
--	293	100
Day -7	630	215
Day -1	360	123
Day 0	873	280

[a] The in vitro primary humoral immune response (Day 5) to SRBC was assessed with cultures established with 5 x 10^6 spleen cells (from mice treated with 0.5 mg $PbCl_2$ on various days prior to removal of the spleens) plus 2 x 10^6 SRBC.

[b] The groups represent two mice per treatment and each spleen was cultured in triplicate.

Our previous analyses of the effects of chronic Pb exposure (Lawrence, 1981c), as well as our analyses of acute Pb exposure in this study, do not provide an explanation for the reduced resistance to extracellular or intracellular parasites (Lawrence, 1981c; Cook et al., 1975; Hemphill et al., 1971; Selye et al., 1966; Gainer, 1974, 1977; Vengris and Mare, 1974; Cook et al., 1974; Figure 5). Resistance to an extracellular parasite needs to be studied in CBA/J mice, because humoral immunity in this strain of mouse does not appear to be inhibited by Pb (Lawrence, 1981c; Figures 2 and 4) whereas humoral immunity has been reported to be inhibited in other strains. CMI (assessed by MLC responsiveness or T-cell mitogen responsiveness) was inhibited slightly by Pb (Lawrence, 1981c; Table 2) but it seems unlikely that this slight inhibition could account for the high degree of mortality resulting from Listeria infection (Lawrence, 1981c; Figure 5) which is considered to be controlled by CMI (Schell and Lawrence, 1977; Mackaness, 1970; North, 1973). Therefore, the ability of Pb to interfere with host resistance to Listeria probably is not due to a direct suppressive effect on B-cells or T-cells. A direct inhibitory effect of Pb on macrophages could interfere with host resistance to extracellular and intracellular parasites. In a previous study, we assessed the in vitro effects of Pb on macrophage binding of SRBC and antibody-coated SRBC and reported that Pb did not inhibit these processes (Lawrence, 1981c). In Table 4, we report that acute in vivo Pb exposure did not alter the binding, phagocytosis, or digestion of SRBC. This type of study needs to be expanded to include antibody-coated SRBC and phagocytic cells from chronically Pb exposed mice, but these preliminary results suggest that Pb also may not directly inhibit

macrophage activities. Previous studies evaluating the influence of Pb on macrophage activities reported that they were inhibited (Bouley et al., 1977), unaltered (Lawrence, 1981c; Koller and Roan, 1977; Table 4), or enhanced (Kamruski et al., 1977). Clearly, more studies of the Pb effects on macrophage activities are needed. Possibly, Pb inhibits host resistance because it interferes with the ability of the products of lymphocytes (B-cell antibodies or T-cell lymphokines) to enhance macrophage activities. These possibilities will be examined in future studies.

TABLE 4. IN VITRO PROCESSING OF ^{51}Cr-SRBC IN PRIMARY CULTURES OF SPLEEN CELLS FROM NORMAL AND LEAD-TREATED MICE

	% Processed[a]				
Cultured Cells	2 hr	6 hr	18 hr	24 hr	48 hr
Normal spleens	5.6	9.9	16.8	20.3	32.1
Day -7 Pb cells	6.9	8.6	17.4	22.4	38.2
Day -1 Pb cells	3.7	9.0	21.5	22.2	29.1
Day 0 Pb cells	4.1	6.8	19.0	20.1	30.1

[a] Percent processed includes ^{51}Cr-SRBC bound to the adherent cells (macrophages), the amount phagocytosed, and the amount digested (% lysed). Amount phagocytosed was calculated as described by Mantovani (1975). Amount digested was calculated by determining the cpm (^{51}Cr) present in the soluble supernatant fraction. Spontaneous lysis (48 h) was <15%.

Additional Pb results which need to be resolved include the apparent inability of Pb to inhibit antibody production in CBA/J mice and the overall resistance of this mouse strain to Pb. Chronic exposures presently up to 54 weeks have produced no increase in the morbidity or mortality of uninfected CBA/J mice. As shown in Figure 6, the blood Pb level of orally exposed CBA/J mice (2 and 10 mM) reaches a high concentration already at two weeks, and after >52 weeks of drinking Pb these concentrations were approximately the same. A mean ± S.D. was not obtained for the >52 week, 10 mM groups, because initially we analyzed samples undiluted and the values (>100 µg/dl) were out of the range of our standards. Of late, we have been obtaining the true values for this group, and their range is 104-1000 µg/dl. For comparative purposes, the CBA/J mice injected with 0.5 mg Pb had 117-145 µg/dl on Day +7. Pb analyses were performed by Delves cup atomic absorption spectrometry (Mitchell et al., 1974). In any case, husbandry of the CBA/J mice drinking Pb

for over 52 weeks is conventional, that is, non-pathogen free; however, they are kept in autoclaved cages with sterile bedding and filter tops. Unless these mice are specifically infected, to date, Pb (even a 10 mM dose) does not affect their longevity.

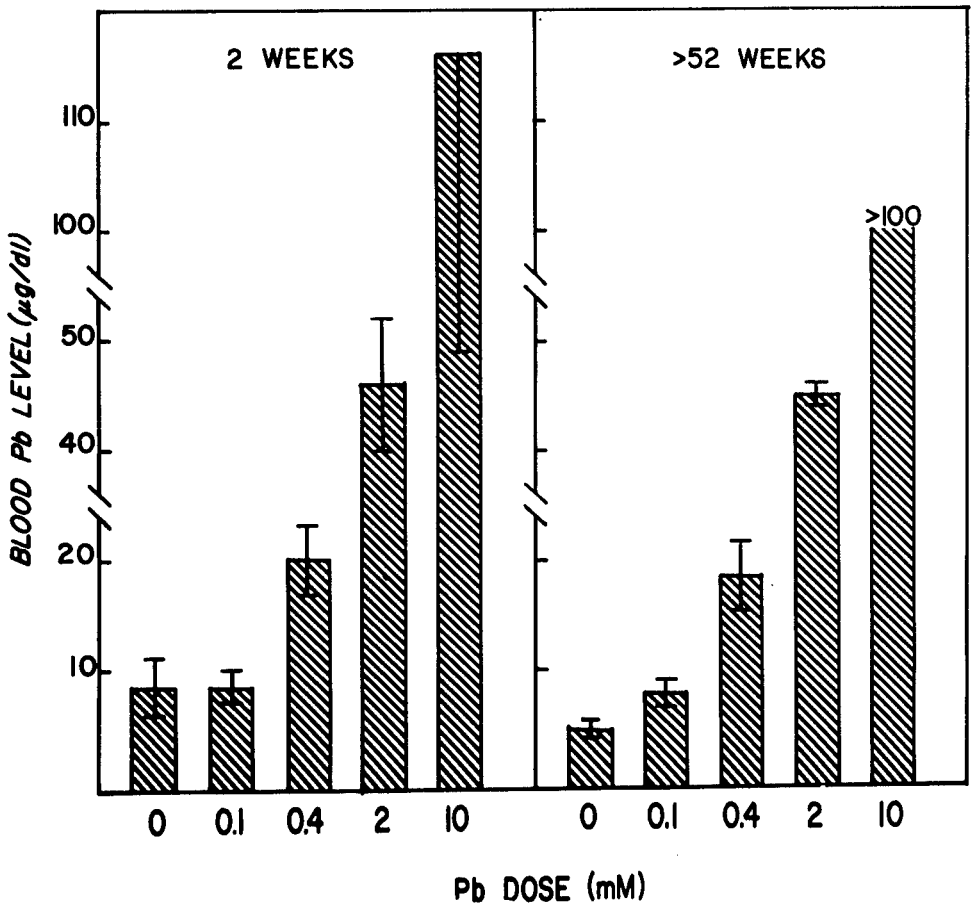


Figure 6. Blood Pb levels. The concentration of blood Pb (µg/dl) was quantitated after 2 and >52 weeks of exposure to various doses of Pb in the drinking water of the mice. Blood Pb was determined by Delves cup atomic absorption spectrometry.

The humoral immunity (HI) and CMI of the CBA/J mice exposed to oral Pb for >52 weeks has been compared to that of mice exposed for four weeks. HI and CMI normally declines with age, and a one year-old mouse usually has a significantly lower response. This age effect is apparent in our results; however, the Pb effects did not differ substantially in 3 month-old mice exposed for four weeks or >60 month-old mice exposed for >52 weeks. The in vitro PFC response (HI) to SRBC of these mice is shown in Figure 7, and 0.1 and 0.4 mM

Pb significantly enhanced, and 10 mM Pb significantly suppressed. As shown in Figure 8, MLC responsiveness (CMI) was inhibited after four weeks with 10 mM Pb only, but after >52 weeks, the 2 and 10 mM Pb dose was inhibitory. Even though the 10 mM Pb dose suppressed the in vitro HI and CMI, in vivo there was no apparent effect on morbidity or mortality, and our previous studies indicated that in vivo antibody production was not inhibited. Therefore, the reason for the suppression by 10 mM Pb when HI responsiveness is assessed in vitro needs to be clarified.

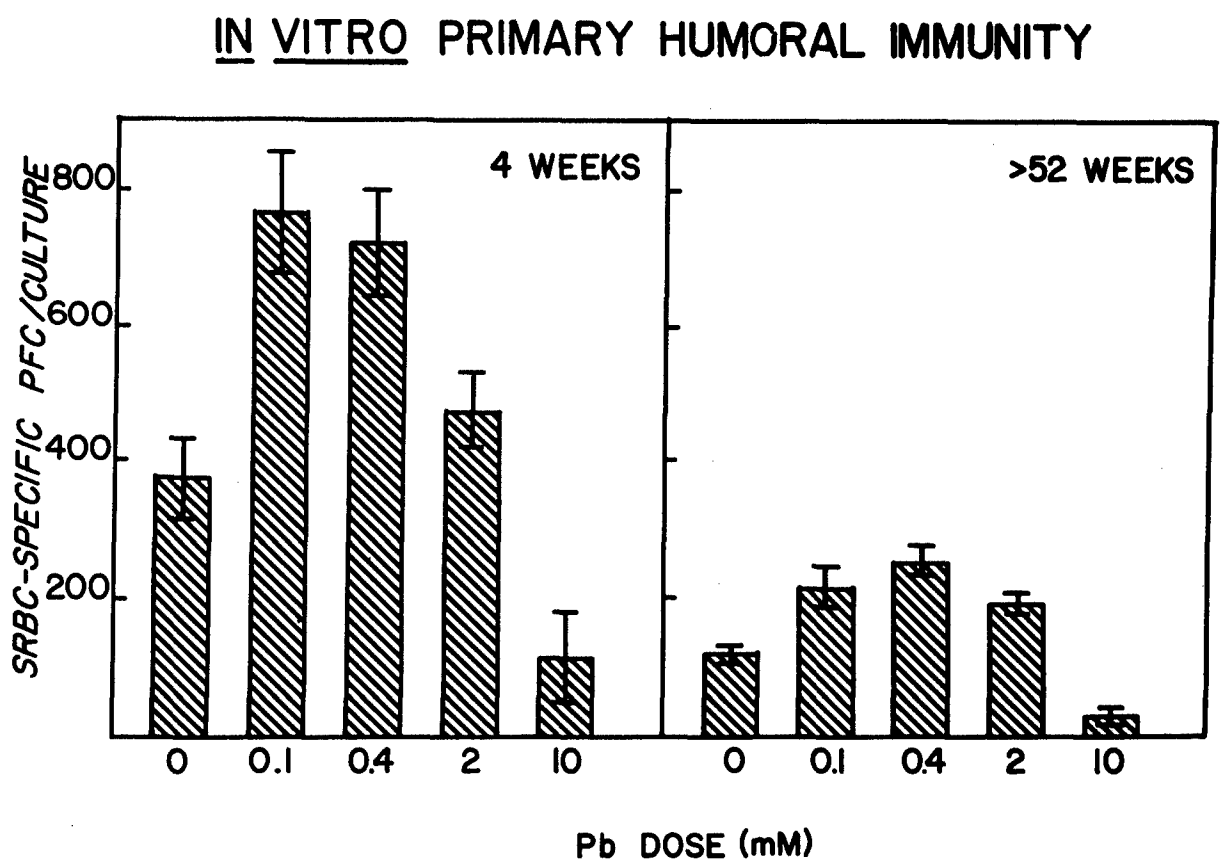


Figure 7. Assessment of in vitro humoral immunity to SRBC by spleen cells from mice exposed orally to various doses of Pb for 4 and >52 weeks. Each bar represents the mean ± S.D. of 3-5 mice/group.

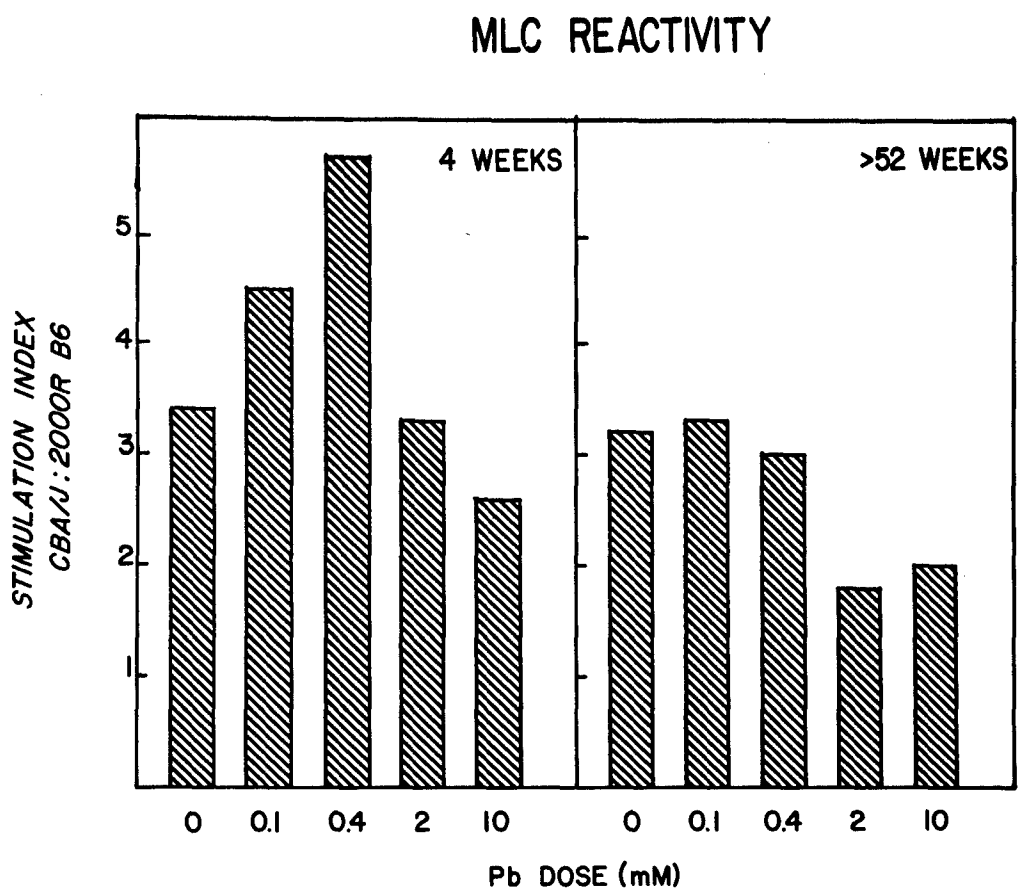


Figure 8. Assessment of in vitro cell-mediated immunity by analyses of MLC reactivity of spleen cells from mice exposed orally to various doses of Pb for 4 and >52 weeks. Each bar represents the mean of two mice/group. MLC reactivity was assessed in triplicate cultures of CBA/J cells and 2000 R irradiated C57BL/6 (allogeneic) spleen cells. Responsiveness was quantitated by incorporation.

Pb, even after >52 weeks, did not induce any renal pathology or immunopathology which was unexpected, since Pb is known to produce pathophysiologic effects on the kidney (Goyer and Rhyne, 1973). The kidneys of the mice were examined by conventional histopathologic and immunofluorescent techniques. Both treated and untreated CBA/J mice had similar mild renal histologic changes resembling the findings in most aging mice. There was a slight increase in glomerular size and mesangial matrix; the tubules appeared normal but there were occasional hyaline casts in the tubules of the medulla; there were focal perivascular accumulations of mononuclear cells around small arteries (Figures 9A-C). The immunofluorescent observations showed that, as in all strains of aging mice, there were glomerular mesangial deposits of IgM, and C3 (Figures 10A,B); the hyaline casts

stained also. These histo- and immunopathologic observations of CBA/J mice were compared to the findings in the kidneys of lupus-prone (NZB x NZW)F_1 mice and some other murine renal diseases (spontaneous interstitial nephritis, induced autoimmune tubulointerstitial nephritis, and spontaneous amyloidosis and papillary necrosis). Pb exposed mice had no similar problems. These results show that CBA/J mice with chronic exposure to Pb do not develop detectable renal lesions or glomerular immune complex deposits. Conceivably, this strain may be genetically resistant to renal damage by certain environmental factors. Susceptibility differences between different H-2 strains in other experimental models have been suggested (Druet et al., 1977; Rudofsky et al., 1980). Thus, it may be that CBA/J mice are relatively resistant to the effects of lead on the kidneys, as well as overall morbidity.

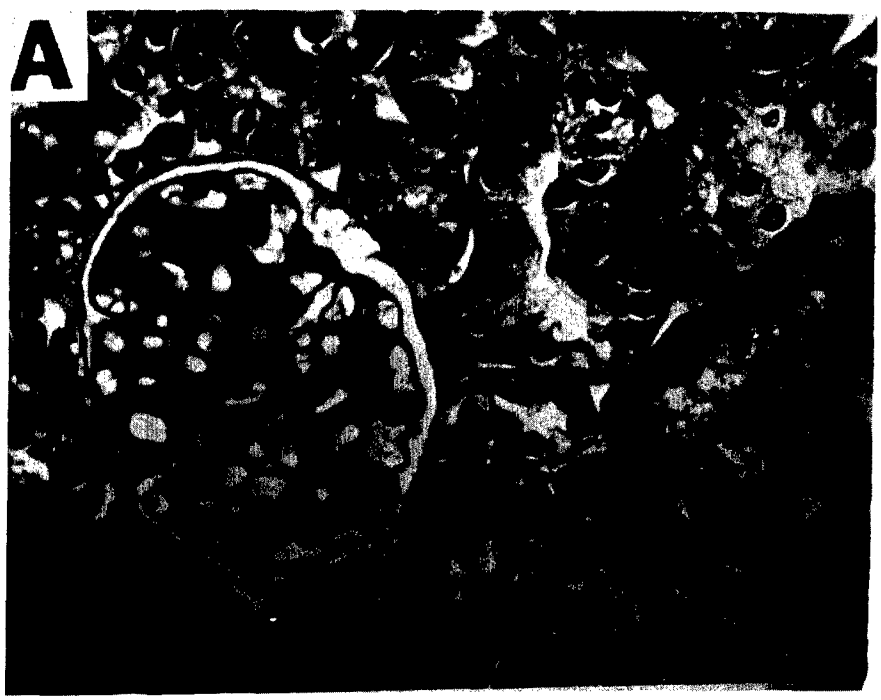
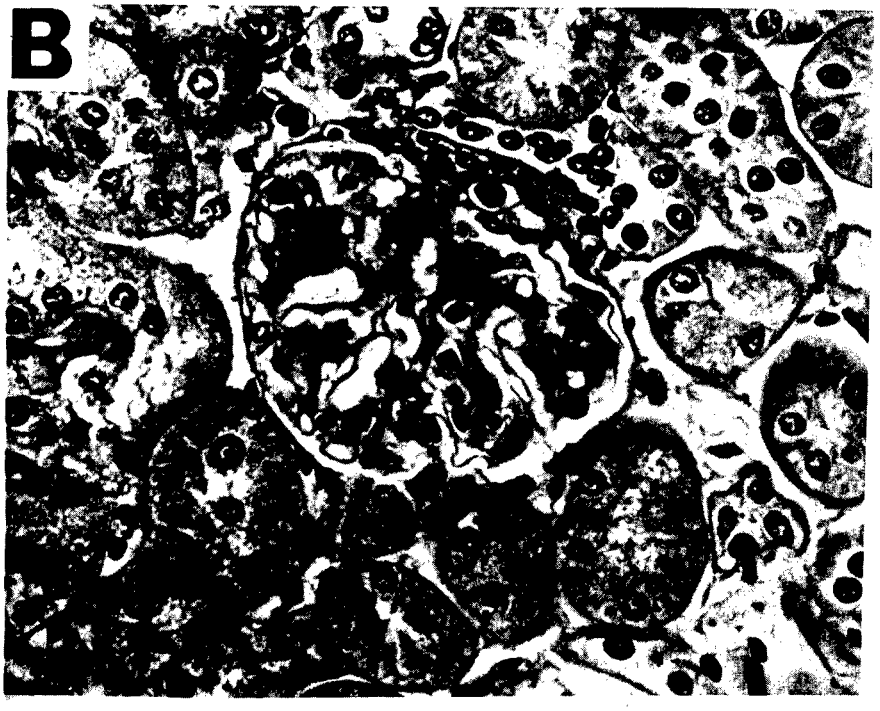


Figure 9. Renal histology of Pb treated CBA/J mice. **A.** Glomerulus of 16 months of age control; **B.** Glomerulus of mouse (16 mos of age) treated with 10 mM Pb for 14 mos; **C.** Kidney papilla of control, untreated mouse. No significant glomerular or rubulointerstitial abnormalities were evident. (Magnified X 780)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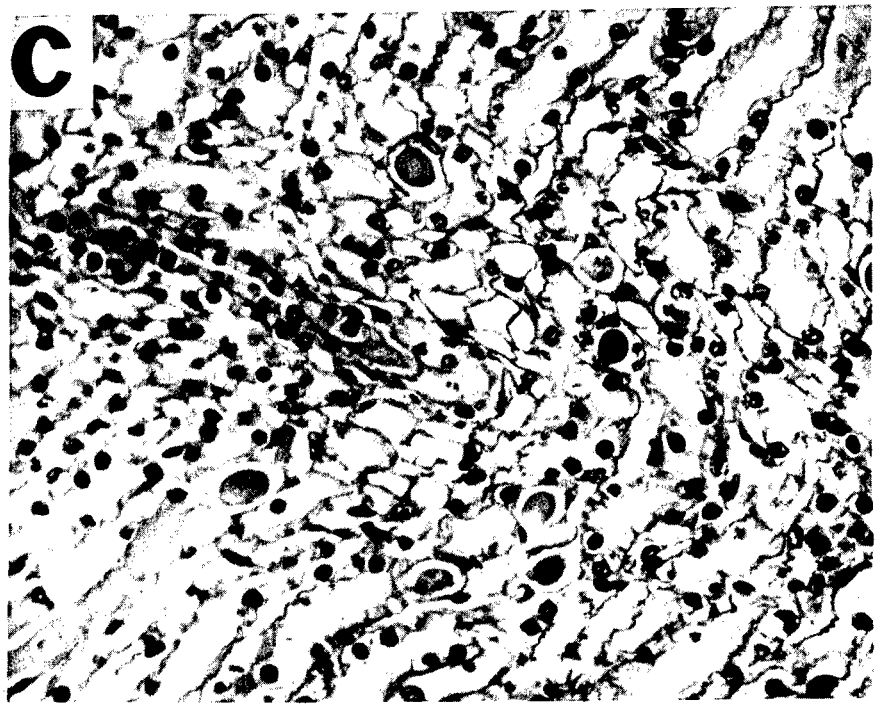
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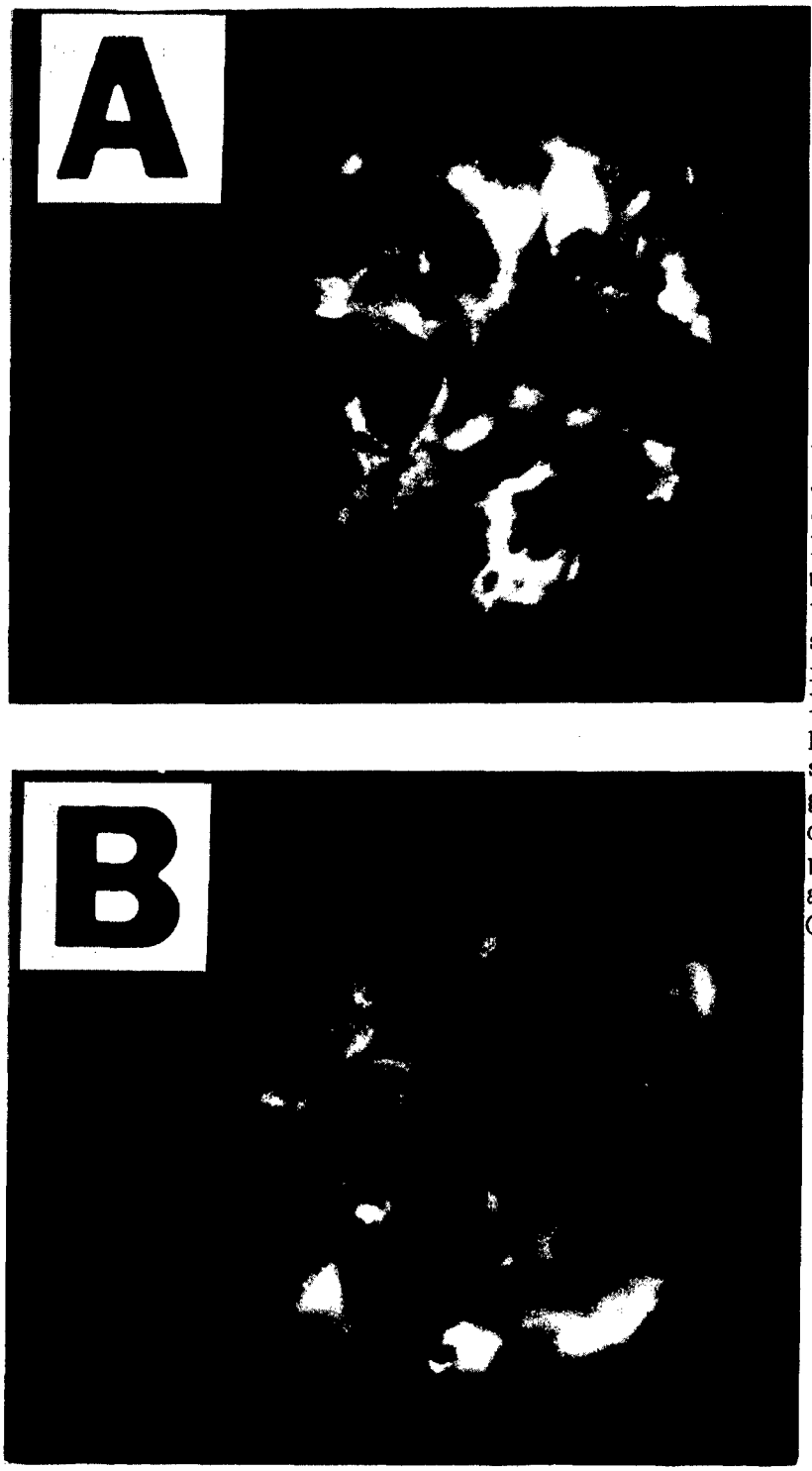

Figure 10. Immunofluorescence of CBA/J kidney sections. **A.** Glomerulus of untreated mouse (16 mos of age) stained with fluorescein-rabbit anti-mouse IgM; **B.** Glomerulus of mouse (16 mos of age) exposed orally to 10 mM Pb for 14 mos, stained with fluorescein-rabbit anti-mouse IgM. Finely granular and amorphous deposits of IgM are seen in the mesangium of glomeruli; capillary loops did not stain. Staining patterns were similar for antisera to mouse IgG and C3. (Magnified 780x)

CONCLUSION

These studies were designed to assess the in vivo effects of acute and chronic exposure to Pb on in vivo and in vitro humoral immune (HI) responses and cell-mediated immune (CMI) responses. In vivo and in vitro T-cell activities were evaluated by resistance to Listeria monocytogenes and mixed lymphocyte culture (MLC) reactivity, respectively; B-cell activities were quantitated by determination of plaque-forming cells (PFC); and macrophage activity was assessed by quantitating the degree of antigen (^{51}Cr-SRBC), binding, engulfment, and digestion. After acute and chronic in vivo Pb exposure, in vivo HI was consistently unaltered or in one case (0.5 mg Pb, Day 0), enhanced, and in vitro assessed HI usually was enhanced with the one exception of chronic exposure to 10 mM Pb which was suppressive. In vitro CMI (a direct correlate of T-cell reactivity) was inhibited by Pb to variable, but slight degrees (24-39% inhibition). Preliminary data (Lawrence, 1981c; Table 4) also suggest that Pb does not inhibit macrophage activities. Overall, the direct effects of Pb on B-cell, T-cell, and macrophage functions do not appear to completely account for Pb's ability to suppress host resistance. Additional studies are required to analyze Pb influences on macrophage bactericidal activity and the ability of lymphoid factors to aid host resistance.

It should be noted that all of our studies to date have employed CBA/J mice. Our recent evidence on the inability of Pb to induce pathophysiologic effects in this strain suggests that CBA/J mice may be relatively resistant to the effects of Pb. After a >14 month oral exposure, there were no apparent effects on morbidity or mortality unless the mice were injected with a pathogen. The CBA/J mice also had no observable renal pathology. Genetically susceptible and resistant strains of inbred mice may be identifiable and will be useful in analyzing the toxicity of Pb.

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PAPER NO. 7

APPLICATION OF ANIMAL MODELS FOR IMMEDIATE AND DELAYED PULMONARY HYPERSENSITIVITY: CHARACTERISTICS OF DELAYED REACTIONS TO TUBERCULIN PROTEIN

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INTRODUCTION

A method was described recently for monitoring delayed-onset pulmonary hypersensitivity reactions in guinea pigs (Karol et al., 1981). To induce sensitivity, animals were injected with Freund's complete adjuvant. Pulmonary reactivity was demonstrated three to five weeks later by bronchial provocation challenge with aerosolized tuberculin protein derivative (PPD). Because onset of reaction occurred typically 9-12 hr following challenge, it was necessary to challenge animals in the "heads-only" inhalation chamber, then remove them from the chamber immediately following challenge and place animals in individual whole body plethysmographs for long-term monitoring. In this way, guinea pigs were free to move about during the ensuing 24 hr while respiratory responses were being monitored.

However, the transfer of animals from the "heads-only" to the whole body plethysmographs resulted in loss of measurements for about 1 hr. In order to provide continuous measurements of respiratory rate, beginning prior to challenge and continuing for 24 hr, a system was established based on a recent report (Zelenak et al., 1982).

The current study describes characteristics of the continuous respiratory response to PPD in guinea pigs. In addition, experiments were performed to determine the influence of several factors on the pattern of delayed-onset responses. Such factors included: repeated inhalation challenge with homologous antigen, time interval between challenges, and history of previous pulmonary reactions to heterologous antigens. Results indicated that respiratory responses to PPD were influenced by previous exposure only to homologous antigen.

METHODS

ANIMALS

Male, English smooth-haired guinea pigs (Hilltop Laboratories, Scottdale, Pennsylvania) weighing 250-300 g were used for sensitization.

SENSITIZATION AND ELICITATION OF PULMONARY RESPONSE

Animals were sensitized to Freund's complete adjuvant (FCA, Calbiochem) by injection of 50 µl of FCA-saline emulsion (1:1 v/v) into each of four footpads (200 µl/animal, Karol et al., 1981). Three weeks later, animals were placed in individual whole body plethysmographs for inhalation challenge. Challenge was performed by generating known atmospheres of PPD aerosol into a 2.1 liter primary chamber. Four individual plethysmographs (each 2 liter) were connected to the central primary chamber via small bore teflon tubing (Zelenak et al., 1982). Aerosols were generated into the primary chamber by a Pitt #1 generator (Wong and Alarie, 1982). Aerosol was pulled from the primary chamber into the individual plethysmographs at 2 liter/minute for each chamber. Total airflow through the system was 20 liter/minute.

MEASUREMENT OF THE RESPONSE

A Statham PM 197 differential pressure transducer was attached to each plethysmograph to detect changes in pressure with each respiration (Karol et al., 1981). The signal from the pressure transducer was displayed on a Gould 200 oscillograph and additionally fed into a Gould Biotachometer set in the averaging mode. The output of the Biotachometer was displayed on a recorder to monitor the average breathing frequency of each animal.

EXPERIMENTAL

REPEATED INHALATION CHALLENGE, CONTINUOUS RESPIRATORY MONITORING

Initial inhalation challenge of FCA-injected animals with PPD aerosol resulted in respiratory reactions beginning 9-12 hours following challenge (see Figure 1A). Animals were allowed a 2 week rest period, then rechallenged in an identical manner with PPD aerosol. This pattern of challenge followed by 2-week rest was repeated for a total of 4 challenges. Response to second and subsequent challenges followed a pattern different from that seen after first challenge (Figure 1A). Repeated challenge resulted in a response by 2 hours which was maximal at 4-5 hours. In addition, there was more rapid recovery from response when compared to that observed following first challenge. The typical respiratory reactions to first,

second, and third challenges of guinea pigs are shown in Figure 1A. These responses were indistinguishable from those observed previously in animals challenged using a "heads only" chamber (Stadler and Karol, 1982) rather than the whole body plethysmograph. The mechanism underlying the respiratory pattern was investigated in experiments described below.

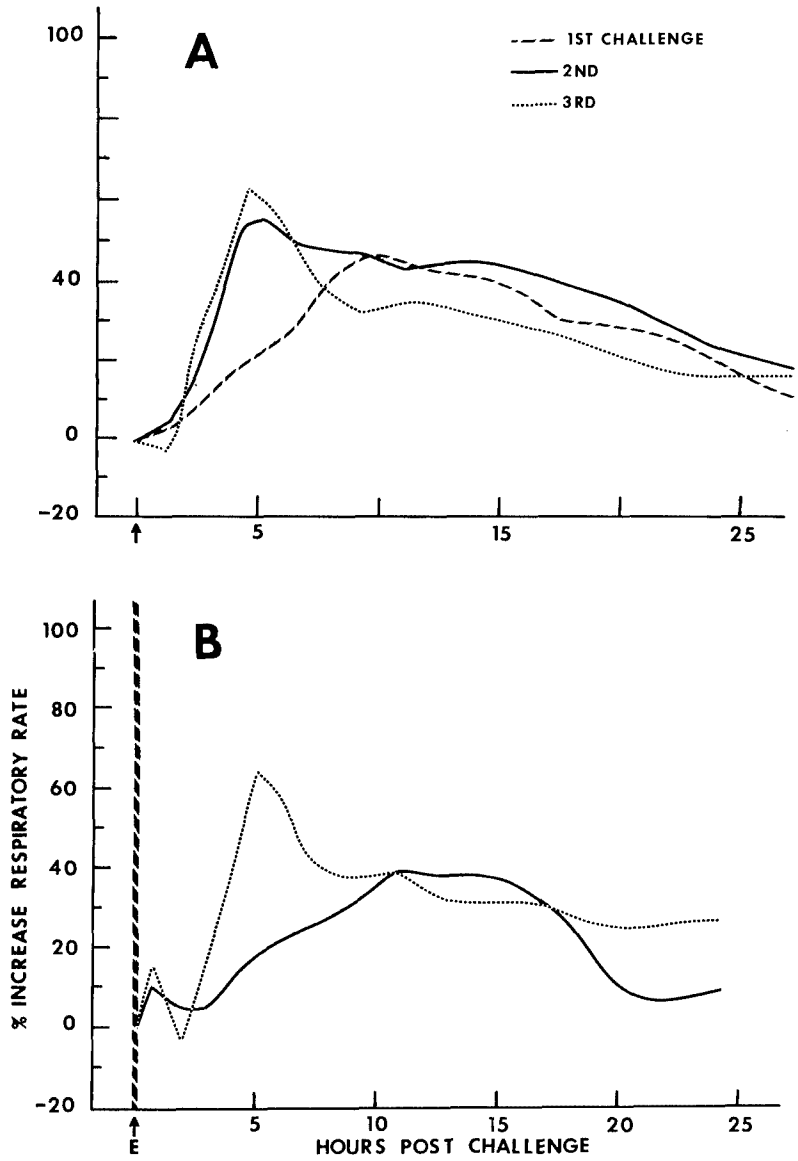

Figure 1. Respiratory reactions to repeated bronchial provocation challenge.

A. Average response of 6 guinea pigs to each of 3 bimonthly inhalation challenges with PPD aerosol.

B. Average response of 6 guinea pigs to OA aerosol (first challenge) followed by bimonthly challenges with PPD aerosol (second and third challenges). E: time of exposure.

EFFECT OF REST PERIOD BETWEEN CHALLENGES

Two groups of guinea pigs were sensitized to PPD by footpad injection with FCA. Three weeks later, all animals were challenged by inhalation of PPD aerosol and displayed the typical delayed-onset pulmonary reactions to first and second challenges (Table 1). Guinea pigs were then divided into two groups. Group A was again challenged with PPD aerosol two weeks following second challenge. This group responded to third challenge with an increase in respiratory rate having maximum intensity 5-6 hours post-challenge. The second group (Group B) was allowed a 6 week rest, rather than the 2 week rest, between second and third challenge. Response of Group B to third challenge was maximal 9-10 hours post challenge. In this regard, it resembled a typical "first challenge" response. Moreover, recovery from third challenge in Group B was prolonged and typical of that seen after first challenge of animals (Figure 1A) rather than typical of third challenge response. The intensity of respiratory responses to each challenge was essentially unchanged and typically averaged 50% (see also Karol et al., 1981; Stadler and Karol, 1982).

TABLE 1. EFFECT OF EXTENDED REST PERIOD ON THE PATTERN OF RESPIRATORY RESPONSE TO PPD INHALATION CHALLENGE

Guinea Pig Group	Time of Response				
	Challenge #1		Challenge #2		Challenge #3
A (N=6)	9-10 hours*	2 week →	5-6 hours	2 week →	5-6 hours
B (N=5)	9-10 hours	2 week →	5-6 hours	6 week →	9-10 hours

* Time of maximal respiratory reaction following challenge.

EFFECT OF ANIMAL AGE ON RESPIRATORY RESPONSE

The possible effect of the age of the animal on the onset of the respiratory response was explored. At the time of initial immunization, guinea pigs weighed 250-300 g. At first challenge they typically weighed 500 g (Table 2). One month later, at third challenge, they were 600 g. In order to determine if maturity (assessed by weight) of the animals at the time of second and third challenge contributed to the earlier response to challenge, the following experiment was performed. A group of guinea pigs was immunized by injection with FCA, and three weeks later, each animal was skin tested with PPD (Karol et al., 1981) to assess sensitivity. Eight animals displaying the most severe skin responses were then divided into 2 groups. Group C was challenged shortly thereafter with PPD aerosol. These animals weighed approximately 500 g at the time of this first inhalation challenge. All animals in

Group C responded to PPD challenge with respiratory reactions 9-10 hours following challenge. Upon second challenge two weeks later, response in these animals occurred 5-6 hours following challenge. Two weeks later, response to third challenge occurred 4-5 hours post-challenge (Table 2).

TABLE 2. EFFECT OF AGE ON ONSET OF RESPIRATORY RESPONSE TO PPD

Weight at Sensitization	Time of Response: Challenge #1	Challenge #2	Challenge #3	Challenge #4
Group C				
250-300 g (N=4)	9-10 hours* (500 g)	5-6 hours	4-5 hours (600 g)	4-5 hours
Group D				
250-300 g (N=4)	---	---	Challenge #1	Challenge #2
			9-10 hours (600 g)	4-5 hours

* Time of maximum response following inhalation challenge.

The second group of animals (Group D) received first inhalation challenge 6 weeks after the skin test. (This was the period of third inhalation challenge for Group C animals). Guinea pigs in Group D were 4 weeks older than those in Group C at the time of first challenge. Group D animals responded to first inhalation challenge with an increase in respiratory rate 9-10 hours following challenge (Table 2). Second inhalation challenge, performed two weeks later, resulted in respiratory reactions 4-5 hours following challenge. It was concluded from these experiments that the earlier response typically seen following second and third inhalation challenges was not caused by maturation of animals but rather was a retest response of the lung. Experiments were then performed to further define the retest response.

EFFECT OF A PREVIOUS RESPIRATORY REACTION TO HETEROLOGOUS ANTIGEN

The possibility existed that as a result of a first hypersensitivity reaction in the lung, a state of "bronchial hyperreactivity" was established which, in turn, affected subsequent pulmonary responses to heterologous inhaled antigens. To evaluate this supposition, the following experiment was performed (Table 3).

TABLE 3. PROTOCOL TO EVALUATE EFFECT OF A PRIOR HETEROLOGOUS PULMONARY SENSITIVITY REACTION ON ONSET OF PULMONARY RESPONSE TO PPD

Day 1	Immunization with Freund's Complete Adjuvant (FCA)
Day 8	Injection with ovalbumin (OA)
Day 22	Inhalation challenge with OA
Day 36	Inhalation challenge with PPD
Day 48	Inhalation challenge with PPD

A set of 6 guinea pigs was immunized by injection with FCA. One week later, animals were injected intraperitoneally with 1 mg ovalbumin (OA) to establish sensitivity to a second antigen. Two weeks following this injection a bronchial response to OA was elicited by bronchial provocation challenge with OA aerosol (Karol et al., 1978). Each of the animals responded within 2-3 minute of challenge with a severe anaphylactic reaction. Animals developed cyanosis and respiratory rates increased by more than 100% (see Figure 1B). This response was similar to that previously observed in guinea pigs sensitized as a result of inhalation exposure to OA (Karol et al., 1978).

Following this first respiratory response, animals were allowed a 2 week rest period prior to second bronchial challenge. For second challenge, however, a heterologous antigen was used, PPD. In this way, distinction was made between second bronchial response to the same antigen (retest response) and second bronchial response where different antigens provoked first and second response.

Results of these repeated challenges are shown in Figure 1. Bimonthly bronchial provocation challenge of guinea pigs with PPD aerosol produced the typical pattern of retest reaction (Figure 1A). First challenge elicited a response 9-12 hours following exposure; second and third challenges with the same antigen resulted in response with both more rapid onset and more rapid recovery.

The response in animals challenged with the set of heterologous antigens is shown in Figure 1B. Initial response to OA was immediate and severe. Second bronchial challenge performed two weeks later elicited respiratory reactions typical of those observed upon first challenge of animals with PPD. Two weeks later, inhalation rechallenge with PPD resulted in a retest response characterized by earlier onset of reaction and earlier wane of response. Results of these experiments clearly indicated that a theory of "bronchial hyperreactivity" could not be implicated as a causative factor for more rapid reaction and recovery observed following repeated challenges with homologous antigen in the lung.

DISCUSSION

Delayed-onset pulmonary reactions have been reported following exposure to a wide variety of environmental and industrial allergens. Reactions are characterized by onset more than one hour after exposure. Frequently reactions occur from 4-24 hours following exposure. Pathogenesis of such reactions remains unclear.

In the animal model described here, repeated inhalation challenge of guinea pigs with PPD aerosol resulted in a pattern of respiratory response different from that seen upon first bronchial provocation challenge. Second and third challenges elicited reactions with both earlier onset and earlier wane of response. This response pattern was produced consistently when animals were rechallenged with PPD antigen at 2-week intervals. However, by extending from 2 to 6 weeks the time betwen PPD challenges, the respiratory response to challenge resembled that seen upon first challenge. These experiments indicated that time between respiratory response affected onset of delayed pulmonary reactions.

Other factors were evaluated for possible effect on time of respiratory response. Varying the age or maturity of animals did not alter the response pattern. Regardless of the age of the guinea pigs, response to first challenge always occurred several hours later than response to subsequent challenges (Table 2).

Several case reports have indicated that with repeated bronchial reactions, onset of response occurred earlier following exposure. The possible development of lung hyperreactivity as a result of repeated pulmonary reactions was evaluated by inducing in guinea pigs a severe allergic pulmonary reaction to ovalbumin. Two weeks following the severe reaction, a pulmonary response to PPD was induced and found to be identical to the response routinely observed upon first PPD challenge of FCA-sensitized animals. Moreover, second challenge with PPD produced the typical PPD-retest response. Together, these experiments led to the conclusion that previous challenge of animals with OA had no effect on the pattern of reactions elicited by PPD.

In summary, earlier onset of pulmonary sensitivity reactions was observed when guinea pigs were challenged bimonthly with homologous antigen. Recognition of factors influencing onset of delayed lung reactions may help elucidate mechanisms underlying delayed-onset reactions observed following exposure to many industrial and environmental agents.

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PAPER NO. 8

IMMUNOSUPPRESSION FOLLOWING EXPOSURE TO EXOGENOUS ESTROGENS

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INTRODUCTION

Physiological estrogens including estradiol and estrone can modulate immune responses. This is evidenced by the fact that female mice possess superior humoral mediated immunity (HMI) and inferior cell mediated immunity (CMI) compared to male mice and these effects can be negated by castration (Ahlquist, 1976). Other studies demonstrating that exogenously administered estrogens and androgens exert negative regulatory influences on CMI and HMI, respectively, support these findings (Fujii et al., 1975; Thompson et al., 1967). These observations may be of particular importance to environmental toxicology since many xenobiotics, natural products and potential environmental pollutants possess estrogenic activity and demonstrate specific binding to estrogen receptors (Katzenellenbogen et al., 1980). While most of these chemicals have relatively weak estrogenic activity compared to estradiol, there is evidence that either chronic exposure or acute exposure to weak estrogens which are not readily excreted (e.g. kepone) may lead to abnormal reproductive development and potential neoplasia (Eroschenko and Palmiter, 1980). Other compounds with weak estrogenic activity include polychlorinated biphenyls, o,p'-DDT, and methoxychlor. Some natural products that demonstrate binding affinity for the estrogen receptor are the flavones genistin, mirestrol, and the fluorescent coumestrol. The mycotoxin zearalanone (P-1502) and its more potent metabolite zearalanol (P-1496) produced by the fungus Fusarium also show remarkable binding affinity for the receptor although fairly poor uterotrophic activity (Katzenellenbogen et al., 1978).

With this in mind we began examining the immunotoxicity of a variety of nonsteroidal and steroidal estrogenic compounds using a

comprehensive testing panel developed at the NIEHS (Dean et al., 1982). Initially, diethylstilbestrol (DES), a nonsteroidal synthetic estrogen with potent estrogenic activity was examined. This compound has been employed as a therapeutic agent in humans as well as a growth promotant in livestock (McMartin, 1978). There is mounting evidence, however, that DES is potentially carcinogenic in humans and laboratory animals and has been associated with endometrial cancer, breast cancer, and vaginal adenocarcinoma (McLachlan, 1980). In mice, DES exposure suppresses specific immunity following either prenatal (Luster et al., 1979)., postnatal (Kalland, 1980a), or adult (Sljivic and Warr, 1973; Luster et al., 1980) exposure. In addition, both estradiol (Seaman et al., 1979a) and DES (Kalland, 1980b) inhibited natural killer cell activity, induced bone marrow myelotoxicity (Boorman et al., 1980), and activated macrophage functions (Boorman et al., 1980). In adult female mice exposed to DES, the effects on specific immune functions are predominantly on CMI rather than HMI (Luster et al., 1980), although this may not be the case following perinatal exposure (Kalland, 1980a). The effects of DES on host susceptibility to infectious agents and syngeneic tumor cell challenge are consistent with these immunological findings since host resistance assays which are dependent upon CMI and macrophage function for primary defense are markedly altered, while those assays dependent upon HMI are not. Thus, mice exposed to DES developed increased numbers of tumors following challenge with transplantable syngeneic tumor cells, increased mortality following challenge with Listeria monocytogenes, and decreased expulsion of adult worms from the gut following infection with Trichinella spiralis (Dean et al., 1980). In contrast to the above assays which are dependent upon CMI and macrophage functions for normal defense mechanisms, parameters which are dependent upon HMI are unaltered in DES exposed mice including susceptibility to infection with Plasmodium (P yoellii 17X) (Hayes, unpublished data) and encephalomyocarditis (EMC) virus (Munson, unpublished observations).

EXPERIMENTAL STUDIES

In more recent studies we have examined the effects of a variety of steroidal and non-steroidal compounds that demonstrate varying degrees of hormonal activity. Zearalanol is an estrogenic metabolite of the mycotoxin, zearalanone, which is produced from a variety of Fusarium species and has been proposed for use as a growth stimulant in certain species of livestock. The metabolite possesses approximately 17% the binding affinity of 17β-estradiol but less than 1% of its uterotrophic activity (Katzenellenbogen et al., 1978). As seen in Figure 1, it contains a phenolic ring structure in the A-ring providing the A-ring region structural similarities to DES and estradiol but not D-ring region. The phenolic ring structure is believed responsible for receptor binding affinity and specificity of estrogenic compounds.

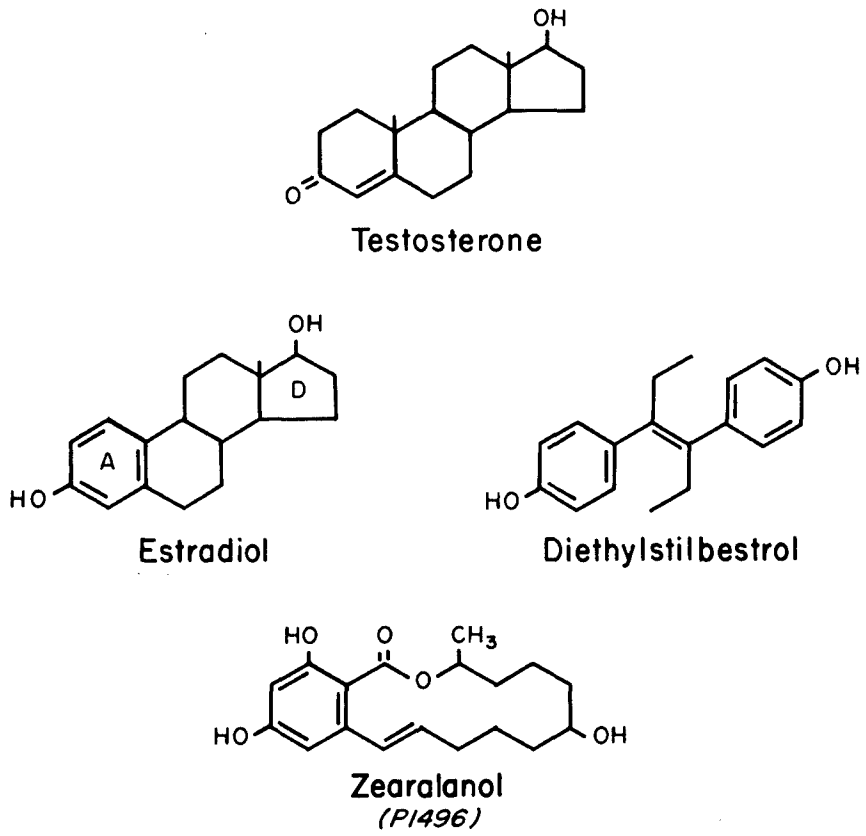

Figure 1. Structural formulae of testosterone, DES, zearalanol and estradiol.

While zearalanol is weakly estrogenic in the uterus, it readily reacts with liver estrogen receptors (Powell-Jones et al., 1981). Testosterone, in contrast, contains D-ring region, but not A-ring region structural similarities to estradiol and DES and appears to be selective for B-lymphocyte functions rather than T-lymphocyte functions (Fujii et al., 1975). It was hoped that by examining structure-activity relationships of these compounds, with respect to the immune system, some insight into the relationship between estrogen-induced immune suppression and hormonal activity would be gained.

For purposes of this presentation only representative parameters of immune function will be presented. Chemically-induced macrophage activation was examined using the growth inhibition assay which determines the capacity of macrophage to inhibit the in vitro growth of leukemia target cells (Dean et al., 1978). In this

instance we examined the ability of plastic adherent peritoneal cells to inhibit the growth of MBL-2 leukemia target cells (20:1 macrophage to target cell ratio). The percent inhibition of target cell proliferation by cells derived from control or chemically exposed mice is represented by ^{3}H-TdR incorporation into target cells and referred to as percent cytostasis. This is calculated from a formula:

$$\% \text{ Cytostasis} = \left(1 - \frac{\text{CPM MBL-2 + MO from treated mice}}{\text{CPM MBL-2 + MO from control mice}}\right) \times 100$$

Lymphocyte functions were determined by quantitating blastogenic responses of spleen cells following activation with T- and B-cell mitogens as well as allogeneic leukocytes. These assays are considered in vitro correlates of immune functions and were described in another chapter (see Chapter by Archer) and in greater detail elsewhere (Luster et al., 1982).

Bone marrow functions were evaluated by quantitating both cellularity and hematopoietic stem cell proliferation as performed in our laboratory (Boorman et al., 1980). Stem cell proliferation was determined by enumerating colony growth following injection of marrow cells into irradiated recipients using the colony forming unit-spleen (CFU-S) assay originally described by Till and McCulloch (1961).

RESULTS AND DISCUSSION

As shown in Table 1, exposure to either DES or estradiol caused severe thymic atrophy while compounds with less estrogenic activity (e.g. zearalanol) had only slight effects. Histologically, the thymus was characterized by severe depletion of cortical lymphocytes in DES or estradiol treated mice. Interestingly, this atrophy is histologically reversible since a normal appearing thymus was evident within 2 to 3 weeks following cessation of exposure to DES (Boorman et al., unpublished data). Cell mediated immunity as represented in Table 1 by lymphoproliferation to the polyclonal T-cell mitogen PHA and to allogeneic leukocytes in the MLC response was suppressed in mice exposed to DES and 17β-estradiol. Estrogenic compound caused a slight increase in LPS-induced B-cell activation, although diminished responses were evident in testosterone treated mice. In this respect, earlier studies indicated that testosterone as well as other androgens primarily affects the B-lymphocyte population as evidenced by depressed antibody plaque forming cell responses (Fujii et al., 1975). The suppression of plaque forming cell responses that we and others have reported following estradiol or DES exposure (Luster et al., 1980; Sljivic and Warr, 1973) represents a delayed onset of peak antibody responses probably induced by increased sequestering of antigen by activated macrophages (Sljivic and Warr, 1973; Bick et al., 1982).

TABLE 1. THYMIC ATROPHY AND PROLIFERATIVE RESPONSES OF SPLENIC LYMPHOCYTES IN HORMONE TREATED MICE

TREATMENT[a]	THYMIC ATROPHY (% CHANGE)	^{3}H-TdR INCORP. (nCPM x 10^{-3})[b] (% CHANGE)		
		PHA	LPS	MLC
CORN OIL	-	34 ± 3	22 ± 3	38 ± 5
DES	79↓**	20 ± 1** (41↓)	25 ± 3 (14↑)	13 ± 3** (66↓)
17β-ESTRADIOL	55↓**	23 ± 3** (32↓)	30 ± 4** (34↑)	20 ± 2** (47↓)
ZEARALANOL	25↓*	33 ± 2 (3↓)	24 ± 2 (9↑)	34 ± 6 (11↓)
TESTOSTERONE	14↓	34 ± 3 (0)	16 ± 4* (27↓)	34 ± 3
PROGESTERONE	4↑	28 ± 2 (18↓)	21 ± 1 (5↓)	ND

[a] Adult female mice were administered 2.8 μmoles of hormone subcutaneously in corn oil over a 5 day period. Each value represents mean ± SEM of 7 mice/group.

[b] Mean ± SEM of 7 mice/group. Data are expressed as net CPM = counts per minute (CPM) in stimulated cultures - CPM in cultures without stimulator.

* $P<0.05$ vs controls.

** $P<0.01$ vs controls.

Table 2 presents bone marrow and macrophage response data in mice exposed to equimolar concentrations of various steroidal and nonsteroidal hormones. As with lymphoproliferative responses, the most pronounced effect on bone marrow and macrophage functions occurs following exposure to the most estrogenic compounds, mainly DES and estradiol. However, zearalanol which as mentioned earlier is considerably less estrogenic than DES or estradiol was equally effective in activating macrophages and suppressing splenic colony forming units (CFU-S) numbers, but had no effect on bone marrow cellularity. Neither progesterone nor testosterone had any

demonstrable effect on bone marrow or macrophage functions. The data in Tables 1 and 2 provide evidence of a chemical disassociation indicating that regulation of estrogen-induced immunotoxicity is a complex event.

TABLE 2. BONE MARROW FUNCTIONS AND MACROPHAGE ACTIVITY IN HORMONE TREATED FEMALE MICE

	BONE MARROW		MACROPHAGE CYTOSTASIS	
TREATMENT[a]	CELLULARITY/ FEMUR x 10^6	CFU-S/ 5 x 10^4 CELLS	^{3}H-TdR INCORP. IN MBL-2 TARGET CELLS (CPM ± SEM x 10^{-3})	PERCENT CYTOSTASIS
CORN OIL	20.5 ± 0.9	16.7 ± 0.3	75 ± 4	-
DES	14.3 ± 1.1* (30↓)	10.2 ± 0.4* (39↓)	29 ± 7*	62%
17β-ESTRADIOL	14.6 ± 0.9* (29↓)	13.5 ± 0.3* (19↓)	30 ± 3*	60%
ZEARALANOL	17.8 ± 1.3 (13↓)	11.6 ± 0.3* (31↓)	34 ± 6*	54%
TESTOSTERONE	17.2 ± 1.3 (16↓)	15.9 ± 0.2 (5↓)	87 ± 7	+18%
PROGESTERONE	17.2 ± 1.4 (16↓)	16.1 ± 0.2 (4↓)	62 ± 9	17%

[a] Adult female mice were administered 2.8 μmoles of hormone subcutaneous in corn oil over a 5 day period. Each value represents the mean ± SEM of at least 6 mice/group.

* $P<0.01$ vs controls.

To determine whether the immunotoxicity was mediated through estrogen receptors, we examined the activity of several estrogen antagonists. Two of the most studied non-steroidal estrogen antagonists, when administered prior to estrogen exposure, are derivates of triphenylethylene and diphenyl (dihydro- or tetrahydro-) naphthalene, represented by Tamoxiphen and Nafoxidine, respectively (Sutherland and Murphy, 1982). These compounds, by themselves, are slightly agonistic but when administered along with estrogens demonstrate partial antagonism with respect to various estrogenic activities by neutralizing the estrogen receptor (Clark et al., 1980). As summarized in Table 3, administration of either Tamoxiphen or Nafoxidine had a significant effect on estrogen-induced thymic atrophy. However, almost complete inhibition of estrogen myelotoxicity and depressed lymphocyte blastogenesis occurred when mice were

pre-exposed to these antiestrogens. We have also recently demonstrated the presence of a specific estrogen-binding component present in relatively high concentrations in the bone marrow as well as confirmed earlier studies by Gillette and Gillette (1978) demonstrating the presence of specific estrogen receptors in thymus cytosol (data not shown). These studies suggest that the initial events associated with immunotoxicity are mediated through a specific estrogen receptor similar to that described in the uterus.

TABLE 3. THE EFFECT OF ANTIESTROGENS ON ESTROGEN-INDUCED IMMUNOTOXICITY[a]

ANTIESTROGEN TREATMENT	ESTROGEN EXPOSURE	PERCENT CHANGE FROM CONTROLS: THYMIC ATROPHY	BONE MARROW CELLULARITY	BONE MARROW CFU-S	LYMPHOCYTE PHA RESPONSE
VEHICLE	VEHICLE	-	-	-	-
TAMOXIPHEN	VEHICLE	40↓**	7↓	2↓	17↓
TAMOXIPHEN	ESTRADIOL	54↓**	5↓	1↓	9↓
NAFOXIDINE	VEHICLE	25↓*	1↓	2↓	13↓
NAFOXIDINE	ESTRADIOL	39↓**	8↓	2↓	6↓
VEHICLE	ESTRADIOL	64↓**	21↓**	18↓**	40↓**

[a] Mice were given 17 μmoles of antiestrogen over a 3-day period. 17β-estradiol (3.8 μmoles) was administered during the last two days of antiestrogen treatment. Mice were tested 4 days later. Each value represents mean ± SEM of 6 mice/group.

* $P<0.05$ vs controls (vehicle).

** $P<0.01$ vs controls (vehicle).

Studies by Stimson and Hunter (1980) suggested that selected immune effects induced by estrogens are mediated through the thymus following estrogen treatment. This was suggested by the fact that sera from estrogen treated rats, but not estrogen exposed thymectomized (Tx) rats, were capable of affecting several in vitro correlates of immune function. In experiments performed in our laboratory, adult female mice were surgically Tx, subsequently exposed to DES or 17β-estradiol and examined in the abbreviated testing panel (Table 4). Unlike non-Tx estrogen treated mice, neither macrophage activation nor suppression of CFU kinetics occurred in DES or estradiol exposed Tx mice. Tx, however, failed to

influence estrogen-induced bone marrow hypocellularity or suppression of lymphocyte function (MLC response). PHA responses of splenic lymphocytes in non-estrogen treated controls were significantly depressed as a result of Tx complicating interpretation of the PHA data. In recent studies, response in sham-Tx, estrogen exposed mice were found to be indistinguishable from estrogen exposed non-Tx mice (data not shown). These data demonstrate that selected immunomodulatory effects induced by estrogens can be relegated to the thymus. Although the manifestations of the estrogen/thymus interplay are obviously complex and probably not responsible for CMI suppression, it is interesting to note that Tx induced a similar bimodal disassociation of immune effects as observed in mice exposed to zearalanol. That is, zearalanol exposure altered macrophage activity and CFU kinetics without affecting lymphocyte blastogenesis or bone marrow cellularity. Tx inhibited the ability of estrogens to influence macrophage activity or CFU kinetics. The molecular events associated with these interactions are unknown but may be initiated by specific binding of the compound to cytosolic receptors present in various target cells (e.g. stem cell) or secondary target organ (i.e. thymus), thus representing a secondary hormonal effect. With respect to the latter, it is well recognized that the thymus is capable of regulating many immune functions including CFU kinetics through "thymic factors", although this regulation usually reflects positive influences (Goodman et al., 1978).

TABLE 4. EFFECT OF THYMECTOMY ON ESTROGEN-INDUCED IMMUNE ALTERATIONS[a]

PARAMETER	NONTHYMECTOMIZED			THYMECTOMIZED		
	CONTROL	DES	ESTRADIOL	CONTROL	DES	ESTRADIOL
MACROPHAGE						
Cytostasis (CPM x 10^3)	63	30*	36*	47	91*	101**
(% Cyto)		(52)	(43)	(75)	(+33)	(+60)
BONE MARROW						
Cellularity (x 10^5)	20 ± 2	12 ± 1**	14 ± 2**	19 ± 1	12 ± 1**	13 ± 1**
CFU-S/5 x 10^4 cells	17 ± 0.3	12 ± 0.2**	11 ± 0.2 **	18 ± 0.1	17 ± 0.3	17 ± 0.3
LP						
PHA (ncpm x 10^3)	25 ± 2	12 ± 2**	16 ± 1**	11 ± 1*	8 ± 1**	12 ± 3**
MLC (ncpm x 10^3)	19 ± 5	6 ± 1**	10 ± 1*	15 ± 1	5 ± 1**	7 ± 1**

[a] Mice were surgically Tx and treated with either DES (4.0 mg/kg) or estradiol (16 mg/kg) two days later for 5 consecutive days. Three days following the last treatment animals were tested.

* $P<0.05$ vs controls.

** $P<0.01$ vs controls.

Mice administered estrogenic compounds demonstrate a marked increase in susceptibility to infection with Listeria monocytogenes (Dean et al., 1980). The mechanisms responsible for the exquisite sensitivity of this system are unknown but appear primarily related to a combination of depressed cell mediated immunity and/or defective bactericidal activity in estrogen-induced inflammatory macrophages (Luster et al., unpublished data). The data from Table 5 suggest that surgical Tx prior to exposure to estradiol is capable of protecting mice from estrogen-induced increased susceptibility to infection. Since surgical Tx protects mice from estrogen-induced myelotoxicity and induction of activated macrophages, it would appear that the effects of estrogens on either or both of these parameters may be at least partially responsible for the decreased resistance to Listeria. Preliminary studies in our laboratory indicate that the mechanism responsible for the estrogen-induced alteration in susceptibility is primarily related to depressed cell mediated immunity and/or defective macrophage bactericidal activity rather than bone marrow myelotoxicity (Luster et al., unpublished data).

TABLE 5. LISTERIA INFECTION IN ADULT-THYMECTOMIZED ESTROGEN TREATED MICE

Treatment	Dead/Total	% Mortality
Sham	0/14	0
Corn Oil	2/13	15
Estradiol	8/13*	62*
Corn Oil ATX	1/13	8
Estradiol ATX	2/14	14

Sham or adult thymectomized (ATX) mice were administered corn oil or 0.1 mg/kg 17β-estradiol daily for 5 consecutive days. Four days following the final exposure all mice were intravenously injected with 5×10^4 viable Listeria monocytogenes.

*Significantly different from corn oil control ($P<0.05$) by Chi-square analysis.

SUMMARY

Exposure to pharmacological dosages of estrogens, including 17β-estradiol and DES, selectively affects immune responses. This immunologic profile is consistent with myelotoxicity, suppression of cell mediated immunity (CMI), and induction of inflammatory macrophages. Modulation of several of these functions is mediated through the thymus, since thymectomy abolishes estrogen-induced macrophage activation, inhibition of CFU-kinetics and increased susceptibility to Listeria infection, but does not inhibit depression of CMI or bone marrow cellularity. These effects can also be

disassociated chemically as zearalanol, an estrogenic mycotoxin, influences macrophage activity and CFU kinetics without affecting CMI. This may be due to structural differences since zearalanol is similar to DES and estradiol in the A-ring region but not D-ring region of the molecule. Underlying this explanation is the demonstration that many of these effects are apparently mediated through estrogen receptors, as indicated indirectly by inhibition with estrogen antagonist and the demonstration of estrogen receptors in thymus and bone marrow cell cytosol preparations. Thus, many of these selective effects may depend upon relative affinity to receptors as well as binding to the relevant target cell(s).

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PAPER NO. 9

EFFECTS OF TOPICALLY-ADMINISTERED PHORBOL DIESTER PROMOTERS ON IMMUNE FUNCTIONS

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INTRODUCTION

Active phorbol diesters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) have potent effects on cell division, regulation and differentiation in vivo (Hecker et al., 1982) and in vitro (Blumberg, 1981). Following topical application to mouse skin, TPA induces acute inflammation (Scribner and Suss, 1978), reflected by erythema, edema, and infiltration of leukocytes. If treatment is continued, inflammation subsides to lower, but chronic levels following three 6 µg applications per week for two weeks. TPA treatment also causes changes in the differentiated state of the epidermis, as witnessed by appearance of increased numbers of basal keratinocytes (dark cells) synthesis of embryonic keratins, and overall resemblance of the epidermis to that found in the fetal state (Hecker et al., 1982). If repeated TPA treatment is preceded by a single dose of a carcinogen such as 7,12-dimethylbenzanthracene (DMBA), at a concentration insufficient to induce tumors alone, multiple tumors (papillomas) of the epidermis appear, a proportion of which are the site for appearance of squamous cell carcinomas.

The above activities of phorbol diesters such as TPA thus led to the question which effects of this agent on cell behavior are sufficient and which are necessary, but insufficient, for its tumor-promoting activity, and which are merely secondary. In this respect the inflammatory activity of TPA, and of all active promoters for the skin, suggests a role for the immune response in this carcinogenesis model. To support this, pretreatment of mice with Bacillus Calmette-Guerin (BCG) has been reported to reduce the tumor incidence in a two-stage mouse skin cancer experiment (Schinitsky et al., 1973). In addition, many reports on interactions between phorbol diesters and diverse cells of the immune system have been published (Hecker et al., 1982). Despite the latter, however, little work has been done to establish the response of the immune

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system to active phorbol diesters administered as in a traditional two-stage carcinogenesis model. As a result, experiments were initiated in this laboratory to examine the immune effects of TPA and other promoters topically administered to mouse skin.

INDUCTION OF SPLENOCYTE MITOGENESIS BY PROMOTERS

We have previously reported (Fish et al., 1981) that active phorbol diester promoting-agents are comitogenic for murine lymphocytes treated with T or B cell mitogens in vitro, while not being mitogenic per se. Following topical administration of 6 μg TPA to the dorsal skin of female 8 week old Balb/c mice, however, this agent was found to be a potent mitogen for spleen cells, as measured by a 4 hour incubation of cell suspensions with ^{3}H-thymidine in vitro following promoter treatment. Skin treatment with 6 μg TPA, a dose typically used in two-stage carcinogenesis protocols, led to a doubling of radiolabel uptake in treated cells in comparison with controls 2 days following administration, followed by a return to control levels by 4 days. A subsequent second TPA treatment led to a markedly enhanced effect, with treated cells showing levels of radiolabel uptake 4-5x those of controls. The peak of radiolabel uptake was again maximal 48 hours after TPA treatment. The response to TPA was found to be dose-dependent, the optimally effective dose being 14 μg. Higher doses had sharply reduced activity.

Treatment of mouse skin with TPA for extended periods results in severe inflammation initially but subsequent partial resolution to a lower level. In analogous fashion, the mitogenic effects of TPA on spleen cells of mice treated twice-weekly with promoter subsided after the first week of treatment but remained significantly elevated following promoter treatments for up to eight weeks.

Topical administration of phorbol esters with less promoting activity than TPA led to stimulation of spleen cell mitosis to correspondingly lesser degrees (Table 1). In this respect phorbol didecanoate and phorbol dibenzoate induced progressively lower effects than TPA. Mezerein, a weak promoter in vivo, but a potent inflammatory agent, showed comparable activity to TPA. Mezerein has been reported to be active in the second state of a two-stage promotion model (Klein-Szanto et al., 1980), however.

INTERACTION BETWEEN TPA AND A SPECIFIC T CELL MITOGEN

When added directly to mouse spleen cell cultures in vitro, TPA is not mitogenic but considerably enhances the mitogenic response to lectins and other lymphocyte mitogens. In contrast, TPA was not found to be comitogenic with the T cell mitogen Staphylococcal enterotoxin A (SEA). Following topical administration or i.v. administration, respectively, TPA and SEA were both mitogenic for spleen cells. When administered in concert, however, the observed

TABLE 1. STIMULATION OF SPLEEN CELL MITOGENESIS BY TPA AND RELATED COMPOUNDS

Compound	^{3}H-Thymidine Uptake[a]	Percent Change	P values[b]
Experiment I			
Acetone	4,610 ± 127		
TPA, 10 µg	30,826 ± 2211	+569	<.001
PDD, 10 µg	22,402 ± 1949	+386	<.001
PDB, 10 µg	14,376 ± 2819	+212	<.001
Experiment II			
Acetone	5,007 ± 586		
TPA, 6 µg	11,508 ± 2829	+130	<.001
Mezerein, 6 µg	14,640 ± 784	+192	<.001
4-O-methyl TPA, 40 µg	5,619 ± 671	+ 12	< .05

[a] DPM/0.5 x 10^6 cells (± SEM)
[b] from analysis of variance, using Scheffe's test to compare means

effect was the sum of the individual effects, suggesting that the two agents were affecting different cell populations. As observed with other lymphocyte mitogens, the response to SEA in vitro was synergistically enhanced by phorbol diesters.

IDENTIFICATION OF CELL TYPE RESPONDING TO TPA

Experiments with lymphocytes in vitro have shown that B cells, T cells, and macrophages are responsive to phorbol diesters. In addition, human lymphocytes are stimulated to divide by TPA, the responding population apparently being a subset of T cells (Touraine et al., 1977). In order to examine the responding cells in the spleen following topical TPA administration, experiments were carried out to remove specific cell populations from spleen cell suspensions of mice following TPA treatment. Removal of T cells with antithymocyte serum and complement, or anti-Thy1.2 antibody plus complement led to no reduction of radiothymidine incorporation, suggesting that T cells were not the target of promoters in this case. The absence of effect on antibody synthesis by B cells following TPA treatment also suggested a lack of effect on B cells. Passage of splenocyte suspensions over a G10 Sephadex column, however, removed the mitogenic response in TPA-treated animals but not in SEA-treated mice, suggesting that cells of the monocyte lineage, or possibly some other leukocyte subgroup retained by the column, were involved.

MODIFICATION OF PROMOTER EFFECT BY IN VIVO INHIBITORS OF TUMOR PROMOTION

In the two-stage model of skin carcinogenesis, several types of agent are able to effectively inhibit tumor incidence. These include retinoic acid (RA), fluocinolone acetonide (FA), and tosyl-phenylalanyl chloromethyl ketone (TPCK). In the three-stage model of carcinogenesis, where promotion can be effected by the sequential actions of TPA and mezerein, TPCK is specifically an inhibitor of the first stage of promotion, RA a specific inhibitor of the second, and FA an inhibitor of both stages (Slaga et al., 1980). Within these three agents FA only is also a potent inhibitor of inflammation.

When topically administered to mouse skin, retinoic acid (1-10 µg), TPCK (10 µg), or FA (1 µg) slightly enhanced mitogenesis in spleen cells. Upon coadministration with TPA, RA showed additive effects on splenic mitogenesis, coincident with a small inflammatory activity. TPCK coadministration resulted in little effect on TPA mitogenesis, whereas FA showed strong inhibitory activity (Table 2).

TABLE 2. SPLEEN CELL MITOGENESIS: INTERACTION OF FLUOCINOLONE ACETONIDE AND TPA

Treatment	^{3}H-Thymidine Uptake[a]	Percent Change	P values[b]
Experiment I			
Acetone	6,622 ± 565		
FA, 3 µg	2,533 ± 512	- 62	0.0001[c]
TPA, 6 µg	14,857 ± 591	+124	0.0001[c]
FA + TPA	5,605 ± 1246	- 15	0.0001[d]
Experiment II			
Acetone	6,299 ± 631		
FA, 1 µg	9,001 ± 233	+ 43	0.0001[c]
TPA, 6 µg	31,632 ± 1296	+402	0.0001[c]
FA + TPA	14,072 ± 3753	+123	0.0001[d]

[a] DPM/0.5 x 10^6 cells (± SEM)
[b] from two-way analysis of variance
[c] probability of no treatment effect
[d] probability of no interaction between treatments

DISCUSSION

The response of splenic leukocytes to phorbol diesters following topical administration was found to be qualitatively different from that observed in vitro. The promoters were mitogenic

following topical administration, but not in cell suspensions. In vivo TPA apparently acted on a cell population other than T cells, whereas those in vitro have been suggested to be a T cell subset. The above findings suggested either that spleen cell cultures in vitro lack necessary accessory cells or factors present for TPA-induced mitogenesis, or that the effects observed in vivo are indirect responses to the promoter, possibly resulting from inflammation. This latter suggestion is also supported by the findings that splenocyte mitogenesis is also induced by mezerein, a weakly-promoting agent but irritating to a comparable degree to TPA. Mezerein, however, does show partial promoting activity in an appropriate animal model and shares many effects with TPA on cell regulation and differentiation. The finding that fluocinolone acetonide (FA) possesses both antiinflammatory and antimitogenic effects in TPA-treated animals also supports a role for inflammation in the induction of spleen mitogenesis by this agent.

The role of inflammation in cancer has been a topic of discussion since antiquity, but the exact mechanism of its involvement still remains unclear. In the two stage model, most, if not all, carcinogens are inflammatory and induce cellular damage. The same is true for all promoters identified to date. Arguments against a specific role for inflammation in promotion have cited the finding that not all inflammatory agents have promoting activity. Some of these agents may be excessively toxic to initiated cells, however, and some, such as mezerein, are nevertheless active in certain stages of promotion.

Taken as a whole, current findings support at least a necessary role for inflammation in tumor promotion. Serious consideration of whether tumor promoters induce specific immunological changes which are directly instrumental in cancer induction must await further detailed study on the effects of these agents on the intact immune system. Studies in this area are currently in progress in this laboratory.

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PAPER NO. 10

QUINONES AND SULFHYDRYL-DEPENDENT IMMUNOTOXICITY

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INTRODUCTION

The work to be described is based on the use of immunologic models to understand mechanisms of chemical-induced toxicity to the lymphoreticular system. We sought to determine structure activity relationships through the study of target cell populations, the identification of toxic metabolites, and the analysis of factors which modulate toxicity.

SULFHYDRYL GROUPS AND IMMUNOTOXICITY

From the literature, it is known that membrane-penetrating sulfhydryl (SH) reagents such as N-ethylmaleimide (NEM) and cytochalasin A are more effective and specific for suppression of cell functions requiring cell surface receptor modulation (Edelman, 1976) and/or subtle shape changes than are SH reagents relatively impermeable to the cell membrane, including 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB) and p-chloromercuribenzene sulfonate (PCMBS). Some of these susceptible cell functions include phagocytosis (and exocytosis) (Elferink and Riemersa, 1980; Mazur and Williamson, 1977; Giordano and Lichtman, 1973; Tsan et al., 1976), blastogenesis (Chaplin and Wedner, 1978), and cell-mediated cytotoxicity (Cerottini and Brunner, 1972; Ralph and Nakoinz, 1980). Accordingly, intracellular SH groups may play a more important role than SH groups associated with ectoenzymes such as ATPases, nucleotide cyclases, and proteases which are also implicated in the regulation of these processes.

Suppression of blastogenesis by SH reagents does not involve changes in lectin-binding to the cell surface (Chaplin and Wedner, 1978; Greene et al., 1976; Berlin and Ukena, 1972; Sachs et al.,

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1973) and studies have repeatedly demonstrated that inhibition of phagocytosis and blastogenesis occurs at concentrations of disruptive agents which do not result in intracellular decreases in reduced glutathione (Chaplin and Wedner, 1978; Lagunoff and Wan, 1979), energy production (Mazur and Williamson, 1977; Lagunoff and Wan, 1979; Chakravarty and Echetebu, 1978; Pfeifer and Irons, 1981) or loss of membrane integrity (Elferink and Riemersa, 1980; Pfeifer and Irons, 1981). It is also known that colchicine must enter the cell to affect cell surface receptor movement (Aubin et al., 1975). Work from our laboratory suggests that particularly reactive SH groups on microtubules represent an intracellular target for SH alkylating agents like NEM and cytochalasin A which contain α,β-unsaturated carbonyl groups. We suggest that the benzene metabolite, p-benzoquinone (p-BQ), produces its immunotoxic effects via the same mechanism (Pfeifer and Irons, 1981; Irons et al., 1981; Pfeifer and Irons, 1982).

CELL-CELL INTERACTIONS AND THE CYTOSKELETON

Lectin-induced blastogenesis, as well as the development of immune responses, is dependent upon cell-cell interactions (Figure 1). Therefore, cell density in culture will influence these responses. In addition, non-lymphoid accessory cells, like macrophages, are also involved in the regulation of the final response (Rosenberg and Lipsky, 1981; Yoshinaga et al., 1972; McClain and Edelman, 1980; Suthanthiren et al., 1980). Cell-cell interactions are not only involved in the afferent arm of the immune response, but the appropriate apposition of cell surface structures, for example, specific receptors for sensitizing determinants and/or gene products of the major histocompatibility complex are also required for expression of lymphocyte-mediated cytotoxicity. This "matching" of cell surface structures occurs during the reversible, primary stage of effector cell/target cell interaction (Cerottini and Brunner, 1974; Pearson, 1978). As shown in Figure 2, both immune T cell cytotoxicity and antibody-dependent killing by a non-sensitized effector cell type (ADCC), the K cell, require modulation of cell surface structures and subtle changes in cell shape for lytic expression (Sanderson, 1981; Ryser and Vassalli, 1981). After exposure to appropriate activating stimuli, macrophages can also act as effector cells via either mechanism (Adams et al., 1982).

Other important cytoskeletal-dependent processes during the amplification of an immune response include phagocytosis, cell motility, and secretion. The cytoskeleton is also involved in the normal function of processes associated with a variety of other specialized cell systems particularly vulnerable to chemical toxicity. Some of these sensitive processes include secretion of hormones by endocrine organs, chemical transmission at the synaptic cleft, morphogenetic interactions during embryogenesis, and spermatogenesis.

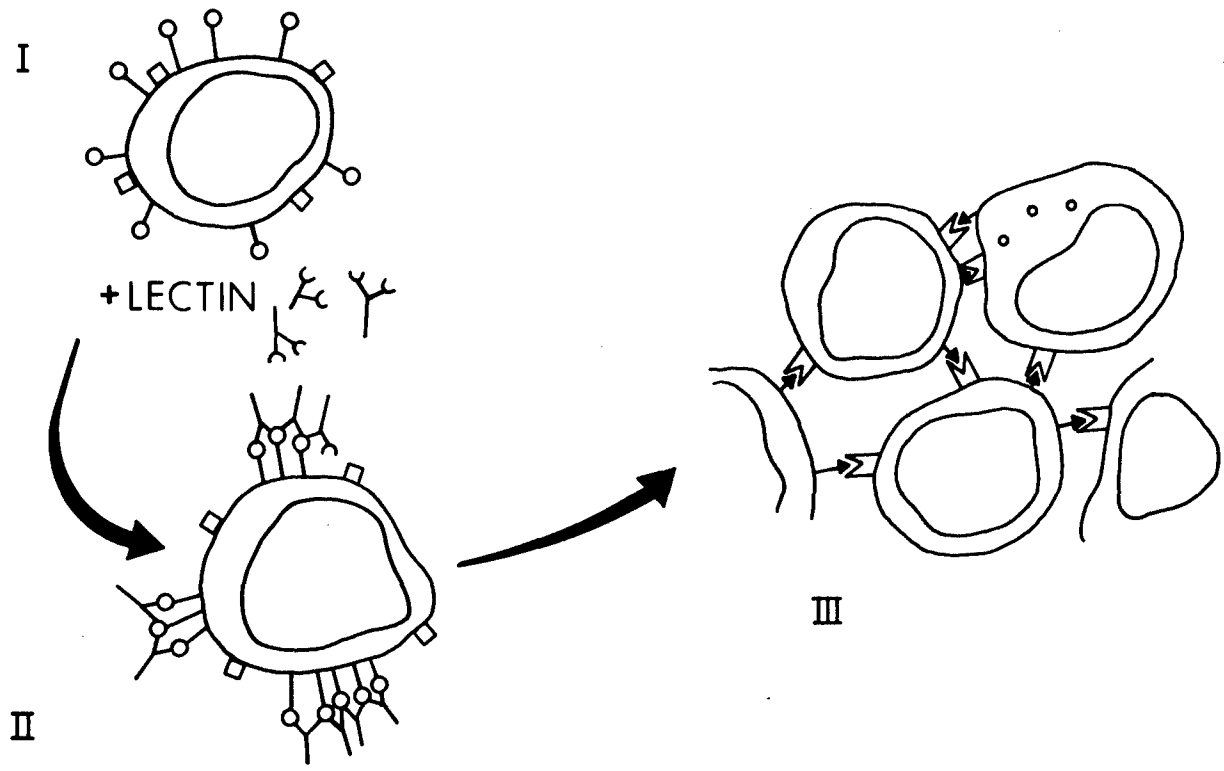


Figure 1. Stages of lectin-induced lymphocyte activation: I "Resting" lymphocyte population. II Transduction of the initiating signal - lectin dependent; cell-cell independent. III Induction of cell-cell communication - lectin independent; cell-cell dependent.

LYMPHOCYTE RESPONSE AND THE CYTOSKELETON

Cell growth and recognition in general are controlled through an assembly of interacting structures at or near the cell surface (Edelman, 1976). Cell surface receptors, glycoproteins in the case of lectin stimulation, extend through the lipid bilayer in random states of attachment with these structures known as microfilaments and microtubules, collectively referred to as the cytoskeleton. That the cytoskeleton modulates membrane receptor mobility can be shown by experiments measuring patch or cap formation after cross-linking cell surface receptors with a polyvalent ligand such as lectin or antibody (Figure 3). Microfilaments and energy are required for the capping phenomenon to occur, but microtubules appear restrictive to the process in that microtubule-disrupting agents will relieve the suppression of capping that occurs with excessive cross-linking of surface receptors. That benzene metabolites might act as microtubule-disrupting agents was suggested by experiments wherein μM concentrations of p-BQ appeared as

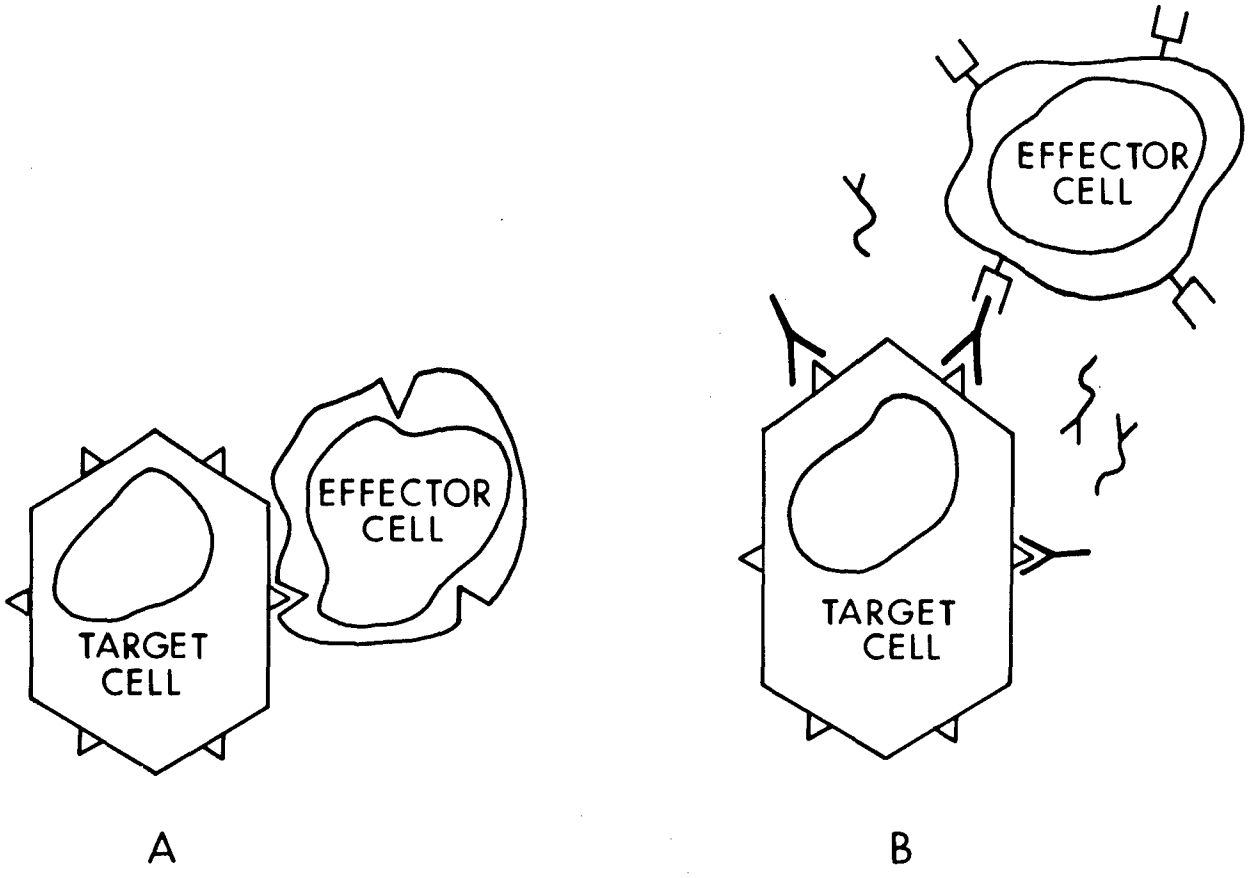


Figure 2. Cytotoxic lymphocyte effector cell-target cell interactions: A. Sensitized T effector cell demonstrates specific receptors in the outer membrane which react with determinants on the target cell. B. Null lymphoid effector cell implicated in antibody-dependent cellular cytotoxicity (ADCC), the K cell, demonstrates no specificity for the target cell, but is activated by the Fc portion of immunoglobulin G after antibody binds to specific target cell determinants.

effective as colchicine at enhancing capping of fluorescent antibody directed against lymphocyte cell surface immunoglobulin in the presence of saturating amounts of lectin (Irons et al.).

BENZENE IMMUNOTOXICITY: EFFECTS OF BENZENE METABOLITES ON A) LYMPHOCYTE FUNCTION AND B) MICROTUBULE ASSEMBLY

Chronic exposure to benzene results in a variety of blood dyscrasias including lymphocytopenia and pancytopenia both in animals and humans; an association with increased risk of leukemia has been made for human exposure (Snyder and Kocsis, 1975). Benzene is not itself considered to be the ultimate toxicant but is

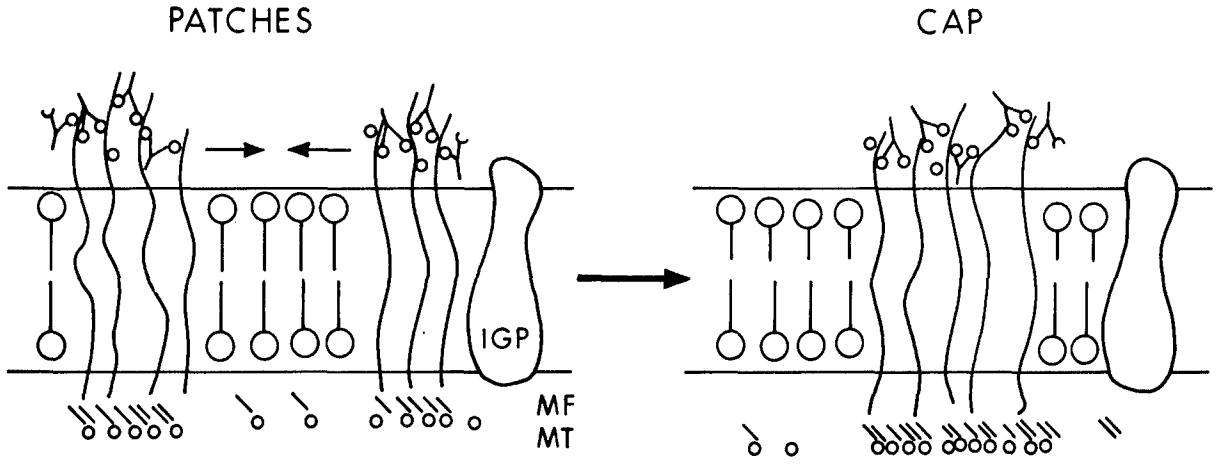


Figure 3. Cell surface receptor anchorage modulation: cell surface receptors span the lipid bilayer variably linked with submembranous microfilaments (MF) and microtubules (MT). PATCHES-crosslinking of receptors with a multivalent ligand such as lectin or antibody results in an alteration in the equilibrium of free versus anchored receptors. The result is an alteration of receptor mobility and effective distribution of receptor sites on the cell surface. CAP-with time, aggregated receptors sites coalesce to form a polar cap. IGP-intramembranous globular protein remains fixed despite alterations in linkage of adjacent receptor sites.

metabolized in vivo by the cytochrome P-450-dependent monooxygenase system to toxic intermediates (Figure 4). Previous work has consistently indicated a correlation between benzene-induced lymphocytopenia and immunotoxicity and the accumulation of hydroquinone (HQ) and catechol (CAT), but not phenol, in bone marrow and lymphoid tissues (Irons et al., 1981).

A) LYMPHOCYTE FUNCTION

The effect of HQ and p-BQ on phytohemagglutinin (PHA)-stimulated lymphocyte blastogenesis is shown in Table 1. Ficoll-purified rat spleen lymphocytes were preincubated with metabolite (usually 30 minutes) and then washed before addition of lectin to cultures. Preincubation with greater than μM concentrations of HQ results in suppression of blastogenesis whereas less than μM concentrations enhances [^{3}H] thymidine uptake to an amount at least twice that observed in control stimulated cultures. Enhancement was

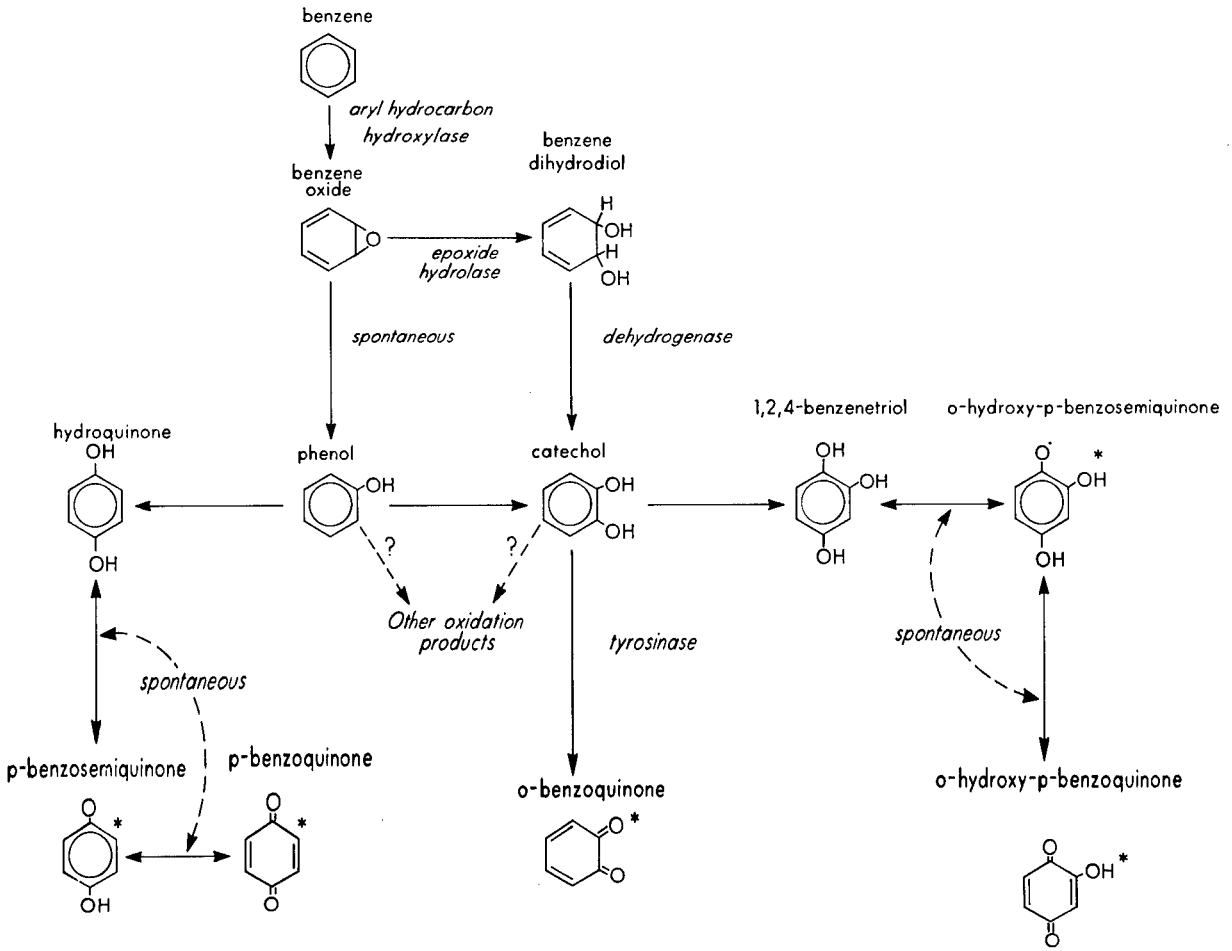


Figure 4. Schematic diagram of the metabolism of benzene demonstrating pathways resulting in the production of potentially reactive metabolites. Asterisks denote puntative or demonstrated alkylating activity toward intracellular nucleophiles.

generally found to be optimal at approximately 10^{-7} M, with stimulation returning to control levels at 10^{-9} M. Experiments wherein a range of lectin concentrations are used demonstrate that enhancement of blastogenesis at relatively higher concentrations of lectin (5.0 µg/ml) was accompanied by a reduction in response at lower concentrations of lectin (2.5 µg/ml) (Pfeifer and Irons, 1982). This type of result suggests that the stimulus threshold for growth response has changed for the responding cell population, allowing an additional response to occur at concentrations of lectin that were previously optimal. Cytoskeletal-disrupting agents such as colchicine and cytochalasin B, and other compounds reportedly giving rise to quinone intermediates such as diethylstilbestrol, have been reported to produce similar modifications of lymphocyte growth response (Yoshinaga et al., 1972; McClain and Edelman, 1980; Suthanthiren et al., 1980). These observations were the basis for developing an in vitro model for detecting chemical effects on differentiating rat bone marrow T cell precursors using flow cytometry to monitor

ontogenetic appearance of specific T cell surface markers (Pfeifer and Irons, 1982; Pfeifer et al.). At any lectin concentration, preincubation with 10^{-5} M HQ results in complete suppression of blastogenesis in the absence of cell death as determined by trypan blue exclusion or ATP production. All the polyhydroxy metabolites of benzene have a similar biphasic effect on PHA-stimulated blastogenesis with p-BQ the most suppressive, approximately twice as potent as HQ (Table 1), followed by 1,2,4-benzenetriol (BT) and CAT. Phenol is not toxic to cultured lymphocytes at any concentration examined.

TABLE 1. EFFECT OF HQ AND p-BQ ON PHA-STIMULATED RAT SPLEEN CELLS[a]

	Hydroquinone		p-Benzoquinone	
Concentration (x 10^{-7} M)	E/C Ratio	A.I.[b]	E/C Ratio	A.I.
4	2.07	++++	0.75	++++
6	1.90	++++	0.72	++++
8	1.56	++++	0.38	+++
10	1.36	++++	0.44	+++
20	0.32	+++	0.01	++
40	0.05	++	0.00	-
60	0.03	+	0.01	-
80	0.03	-	0.00	-
100	0.02	-	0.01	-

[a]Responses of Ficoll-purified cells pooled from F-344 male rats were assayed at optimal time points (48-72 hours after mitogen addition), cell (10^6 cells/ml) and lectin (5 µg/ml) concentrations. Values are expressed as the stimulation ratio of experimental to control (E/C) cpm. Results are calculated from the means of triplicate cultures wherein the S.D. of the mean did not vary more than 10%.

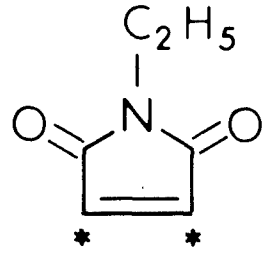
[b]Agglutination index (A.I.) indicates degrees of cell aggregation and blast transformation after exposure to PHA as observed by phase-contrast microscopy: +, appearance similar to cultures with no mitogen; ++ or +++, increasingly larger aggregates of cells, including blasts, and increased numbers of aggregates; ++++, appearance similar to cultures receiving no metabolite pretreatment; -, cells separate and equally spaced with no evidence of aggregation or blast transformation.

It was observed that suppression of lectin-stimulated agglutination occurs in parallel with suppression of blastogenesis (Table 1). Pretreatment with sublethal inhibitory concentrations (10^{-5} M) of HQ or NEM results in complete suppression of lectin-induced lymphocyte agglutination and blast transformation; in culture, the cells appear separate and equally spaced, demonstrating less cell-cell contact than that observed in unstimulated cultures. Although there is not uniform consensus on the importance of cell-cell interactions in the initiation of cell division, agglutination has been reported to be a a prerequisite for blastogenesis and certainly represents one of the earliest events associated with cell division (Wedner and Parker, 1976). Spectrophotometric quantitation of PHA-induced lymphocyte agglutination suggests that the increased adherence properties of lymphocytes occurring within minutes of exposure to lectin is inhibited concomitantly with blastogenesis by μM concentrations of membrane-penetrating SH alkylating agents; more interesting, the agglutination phenomenon is enhanced at the same low concentrations (<μM) of the agents which result in augmentation of blastogenesis as measured by [^{3}H] thymidine uptake several days later (Pfeifer and Luster, 1983).

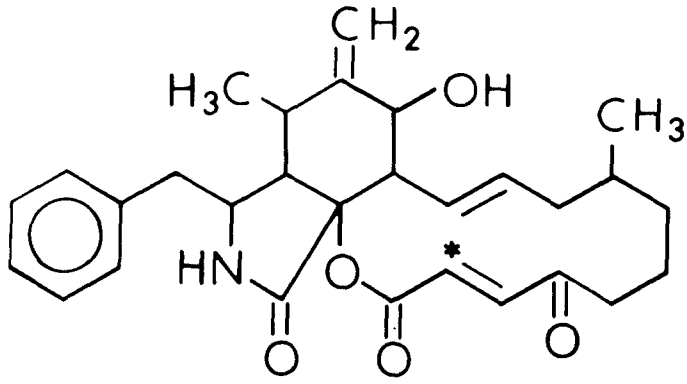
Microtubules, important in the mediation of lectin-induced agglutination (Berlin and Ukena, 1972), have been suggested to be a requirement for transduction of the initiating signal for blastogenesis (Edelman, 1976; Greene et al., 1976; Gunther et al., 1976; Sherline and Munday, 1977; Wang et al., 1975). This does not necessarily require that the two events, signal transduction and agglutination, occur at the same point in the commitment to blastogenesis. The relationship of agglutination to blastogenesis may be explained by events happening somewhat later than mitogenic signal transduction.

The structures of NEM, cytochalasin A and p-BQ all feature a highly polarized, unsaturated carbon-carbon bond (carbonyl electron-withdrawing groups on either side) which is subject to attack by highly reactive SH groups acting as nucleophiles; a conjugate addition reaction (Figure 5). HQ, which theoretically autoxidizes to the p-BO product, was compared to NEM for effects on cell function. After pretreatment of cells, both HQ and NEM produce a sublethal, concurrent inhibition of lymphocyte blastogenesis and agglutination at the same concentrations. The addition of a SH compound, dithiothreitol (DTT), to the incubation tube with either HQ or NEM protects against the inhibitory effects of both agents in a concentration-dependent manner (Figure 6). Similar effects on cultured cells have been noted in our laboratory for cytochalasin A and p- and o-aminophenol. The latter two compounds are aniline metabolites and are theoretically capable of oxidative conversion to benzoquinoneimine derivatives (Pfeifer and Irons, 1983). However, pretreatment with DTNB, a poorly penetrating SH reagent, failed to inhibit either agglutination or PHA mitogen response (Figure 6). These findings are consistent with the selectivity of HQ and NEM for SH groups relative to other nucleophilic groups. Although cysteine

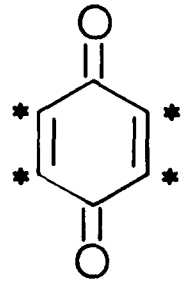
affords complete protection, preincubation with mM lysine, serine, and imidazole at physiologic pH fail to protect against toxicity by these SH reagents. Therefore, it appears that 1) HQ suppresses blastogenic response by interacting with intracellular SH sites and 2) sublethal impairment of immune function by HO is mimicked by a SH alkylating reagent, NEM.



N-Ethylmaleimide



Cytochalasin A



p-benzoquinone

Figure 5. Structures demonstrated to alkylate SH groups at physiologic pH via Michael addition. Asterisks denote carbons subject to nucleophilic attack.

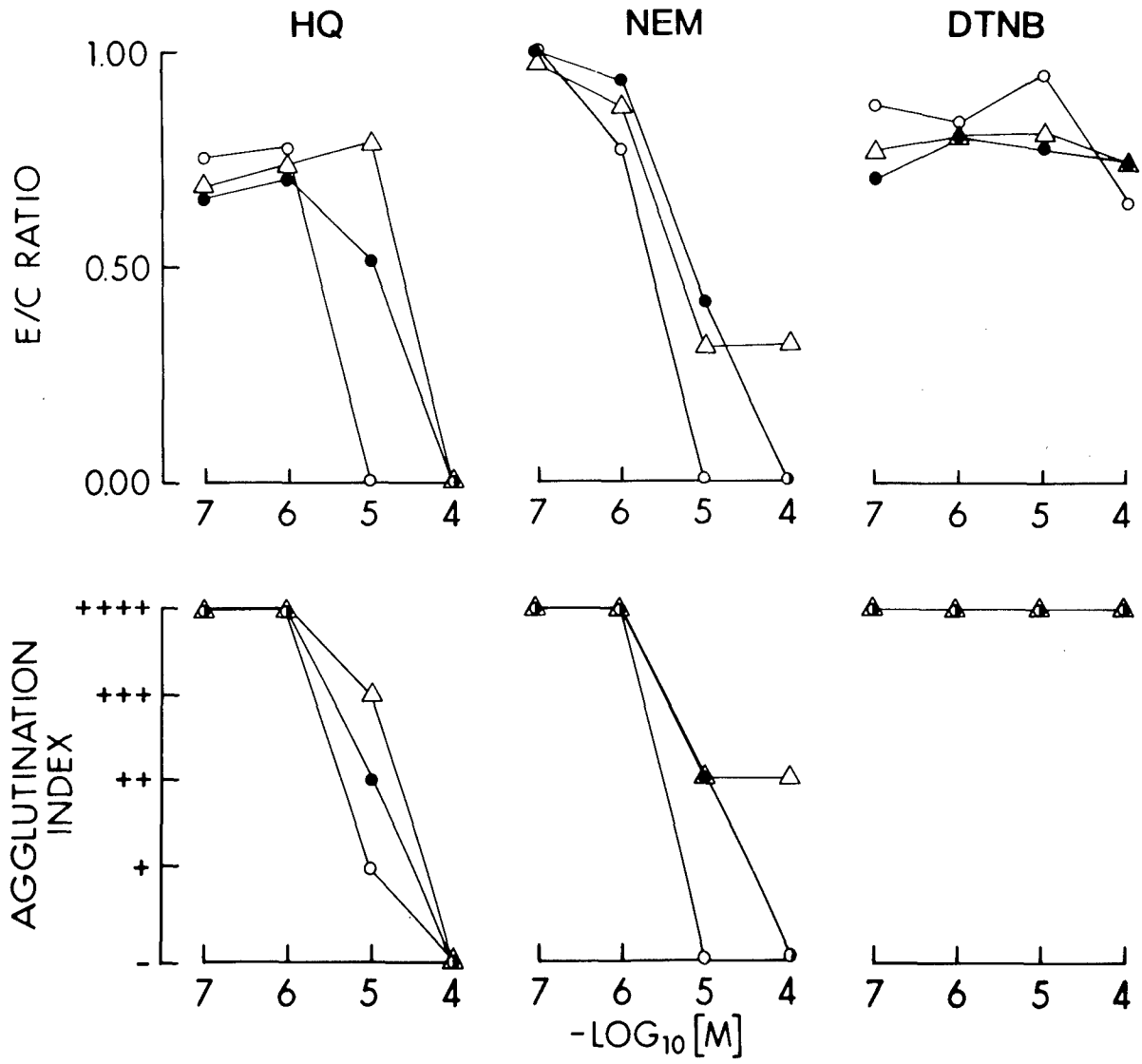


Figure 6. Protection against HQ or NEM inhibition of PHA-stimulated blastogenesis and agglutination in Ficoll-purified rat spleen lymphocytes by DTT. Comparisons of effects of NEM, a cell penetrating SH reagent, with DTNB, a poorly penetrating SH reagent. O, No DTT present during preincubation with HQ or SH reagents; Δ, 10^{-4} DTT present, ●, 10^{-5} M DTT present during preincubation. Results for blastogenesis expressed as stimulation ratio of experimental to control (E/C) cpm. Results are calculated from the means of triplicate cultures wherein the S.D. of the mean did not vary more than 10%. Agglutination index determined as for Table 1.

B) MICROTUBULE ASSEMBLY

The integrity of SH groups on tubulin is a functional requisite for stability. The GTP-binding site on tubulin has been well characterized and involves two out of approximately eleven titratable SH binding sites varying somewhat on the method of isolation. It is known that these two SH groups are statistically more reactive with SH alkylating agents which inhibit microtubule assembly than other SH sites on the tubulin molecule (Ikeda and Steiner, 1978; Kuriyama and Sakai, 1974; Mann et al., 1974; Mellon and Rebhun, 1976).

Cycle-purified rat brain tubulin was isolated to monitor the effects of benzene metabolites on in vitro activity. Temperature dependent tubulin polymerization was measured turbidimetrically at 350 nm following addition of p-BQ, HQ, NEM, or BT at various concentrations. Polymerization was inhibited by all of these agents in a concentration-dependent manner (Figure 7). The stoichiometry of the HQ:tubulin interaction suggests a small number of binding sites (Edelman, 1976; Elferink and Riemersa, 1980; Mazur and Williamson, 1977) are involved in the inhibition of this function (Irons et al., 1981). The linearity of the semilogarithmic plot of inhibition versus the concentration of p-BQ or NEM is characteristic of a first order reaction and suggests a direct reaction of p-BQ and NEM with tubulin to inhibit polymerization; colchicine demonstrates a similar linear plot. Minimally effective concentrations of p-BQ and NEM are the same, although differences in slope of the lines may reflect increased affinity for additional SH sites by NEM relative to p-BQ.

Inhibition of polymerization by HQ and BT is non-linear, indicating the kinetics of inhibition are complex. Other experiments suggest that this reflects the requirement for HQ and BT to be autoxidized to reactive quinone or quinone analogs before reaction with tubulin (Irons et al., 1981). Conversely, anaerobic conditions did not protect against the effects of NEM and p-BQ on tubulin. DTT, but not lysine or serine, protects against the effects of p-BQ and NEM on tubulin, analogous to experiments with whole cells. CAT has no effect on polymerization (5×10^{-4}M); however, if tyrosinase (10 µg/ml) is added to the system, as little as 10^{-5} M CAT results in complete loss of tubulin polymerization. Tyrosinase converts CAT to a highly reactive o-quinone directly via a two electron transfer, suggesting a direct effect of the quinone on tubulin.

HOW DOES THIS MECHANISM FIT IN WITH TRADITIONAL MODELS OF ACTIVATION?

It is known that modulation of intracellular cyclic nucleotides represents an important secondary signal for activation of gene derepression. In many pharmacological models, but especially in the

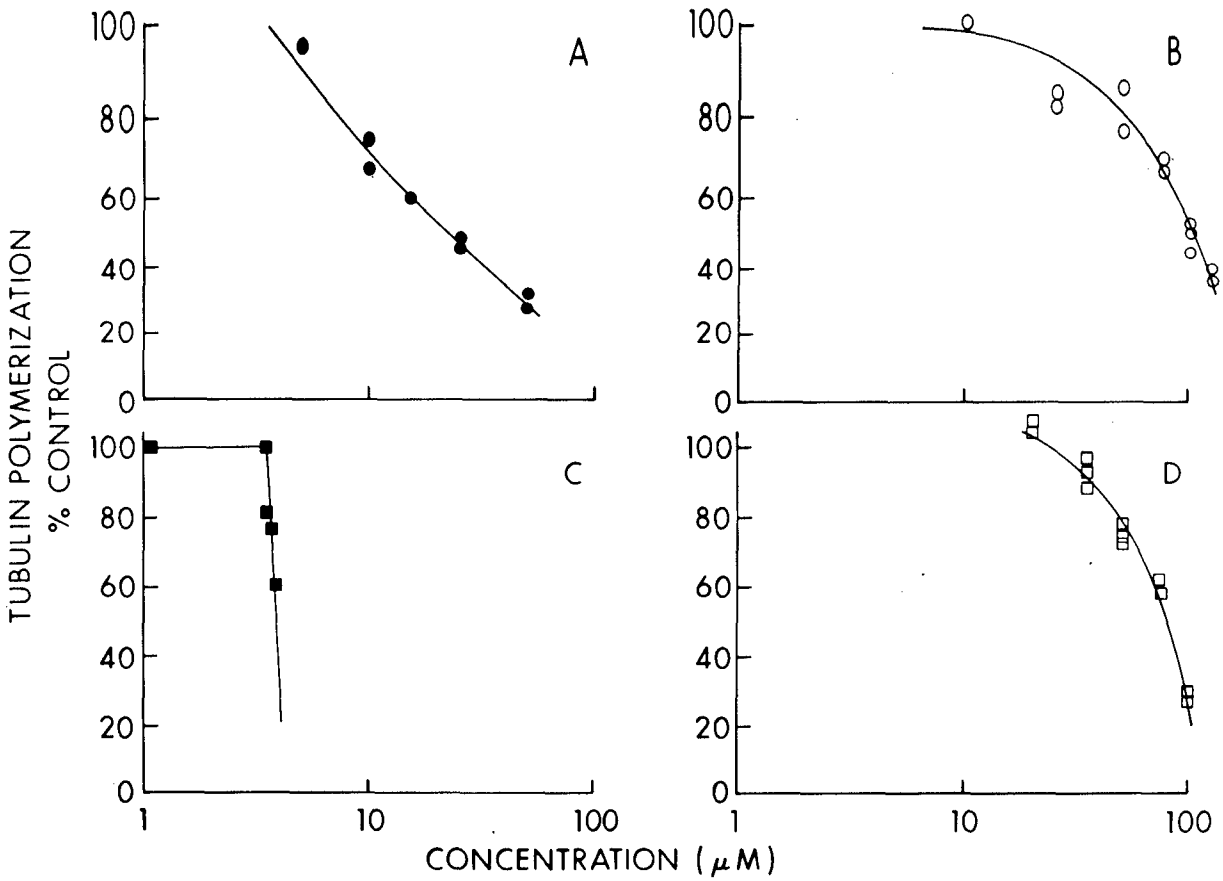


Figure 7. Log concentration-inhibition curves for a) p-BQ, b) HQ, c) NEM, and d) BT on cycle-purified rat brain tubulin polymerization or self-assembly in vitro. Temperature dependent polymerization was measured turbidmetrically at 350 nm after 10 min. incubation at 37°C. Inhibition calculated as percent of control.

area of immunoregulation, there is a satisfying correlation between changes in intracellular levels of these mediators and modulation of cell functions; net increases in cGMP result in enhancement of secretion, cell-mediated cytotoxicity, proliferation and differentiation of immune cells, while net increases in cAMP result in suppression of these functions (Ignarro, 1977; Gillespie, 1977; Strom and Carpenter, 1977; Henney et al., 1972; Lane, 1978; Strom et al., 1972; Hadden, 1977; Watson, 1977). However, it is also true that mobilization of extra- and intracellular Ca^{2+} stores and an intact cytoskeleton appear to be a common denominator for effective functional response to specific initiating signals in the same cellular models (Edelman, 1976; Cerottini and Brunner, 1972; Rosenberg and Lipsky, 1981; Yoshinaga et al., 1972; Sanderson. 1981; Ryser and Vassalli, 1981; Adams et al., 1982; Weissmann et al., 1981; Henson et al., 1981; Keller et al., 1981; Gale and Zighelboim, 1974). Recent evidence demonstrating the intimate association of calmodulin

(Ca^{2+} binding protein), membrane nucleotide cyclases and microtubules (Rasenick et al., 1981; Watanabe and West, 1982) suggests a transducing role for the cytoskeleton which might be susceptible to chemical/drug regulation. For example, microtubule-disrupting agents appear to increase cAMP response in stimulated cells, presumably by influencing the mobility of cell surface receptors and membrane adenylate cyclase activity (Greene et al., 1976; Rudolph et al., 1977).. Alternatively, direct cell-cell contact is a prerequisite for full functional expression of cell activation and might well represent a secondary locus for expression of injury to cytoskeletal structures.

Although we have not looked at the effects of NEM or p-BQ preincubation on other early events of lymphocyte activation such as RNA and protein synthesis, other investigators have demonstrated that colchicine, at concentrations that specifically inhibit DNA synthesis, also significantly inhibits these early biochemical events (Sherline and Mundy, 1977). Since preincubation of lymphocytes with μM NEM or p-BQ results in failure to undergo blast transformation, (Pfeifer and Irons, 1981; Irons et al., 1981; Pfeifer and Irons, 1982), we hypothesize a similar suppressive activity for membrane-penetrating SH alkylating agents, concomitant with inhibition of microtubule assembly. Significantly greater concentrations (0.1-1.0 mM) are required to inhibit plasma-membrane associated regulatory enzymes including guanylate cyclase (Haddox et al., 1978), or those involved in energy metabolism.

CONCLUSIONS

We suggest that early changes in cell agglutination after lectin binding require intact microtubules. These changes, like other early cell responses including increased Ca^{2+} accumulation and cGMP dependent protein kinase levels (Hadden, 1977), occur within minutes. The fact that hormonal and neurotransmitter agents that increase cGMP do so only in intact cells and that activation requires Ca^{2+} suggests an important role for microtubules and calmodulin in the activation of guanylate cyclase. Furthermore, the intimate interrelationship of calmodulin, membrane cyclases, microtubules and phosphodiesterases suggests that effects on one component would be expected to influence the function of the others and that none dominates the functional response of activated cells (Watanabe and West, 1982).

Particularly reactive SH groups on tubulin may constitute an intracellular target, important to normal growth control of the mammalian cell, which are uniquely sensitive to p- and o-quinone metabolites of immunotoxic xenobiotics or analogous resonance structures that possess SH-alkylating activity. Potency differences are probably related to both the efficiency with which oxidation of precursor molecules to reactive quinone structures occurs, and to the SH-reactivity of the quinone or substituted quinone.

The apparent sensitivity of microtubule assembly to SH-alkylating reagents suggests that the process may be susceptible to regulation by similarly reactive endogenous molecules under normal physiologic circumstances. For example, recent work quantitating PHA-induced lymphocyte agglutination spectrophotometrically suggests that the catechol estrogen metabolites are the most potent in suppressing this function, although the parent compound, 17β-estradiol, demonstrates little or no activity (Pfeifer and Luster, 1983).

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Tables 1 and figures 4-7 are reproduced with permission from Plenum Press (1982), Environmental Science Research, Vol. 25 (Genotoxic Effects of Airborne Agents), eds. R. R. Tice, D. L. Costa, and K. M. Schaich, pp. 241-256: Irons, R. D. and R. W. Pfeifer, Benzene metabolites: evidence for an epigenetic mechanism of toxicity.

DR. LUBET (Microbiological Associates): Dr. Sonnenfeld, have you looked for the inhibition of interferon in any compounds that are significantly toxic and non-carcinogenic?

DR. SONNENFELD (University of Louisville School of Medicine): No, we have not looked at compounds of that nature. We know that toxicity is probably not playing a major role in what we are looking at. It wouldn't surprise me if various toxic compounds that were really inhibiting cell metabolism could have an effect on the interferon system and certainly materials such as vinblastine or colchicine have been shown to have such effects. That's why I think it's important to note that if this were ever used as a screening system it would be part of a battery of tests as most screening systems are. I wouldn't expect it to stand alone.

DR. LUBET: Would someone comment on Hewitt's argument that most spontaneously occurring tumors probably do not elicit a significant immune response?

DR. MUNSON (Medical College of Virginia): I think that this is a very true statement. Most human carcinomas spontaneously occurring are to some extent chemically induced. At best, a weak antigenicity is associated with them. I think that this has been the biggest problem with the immune surveillance theory as initially proposed by Burnett. There are other factors that are now coming into play that still have to be looked at such as the natural killer cell and its role. There was a very good editorial written by Dolf Adams in Cancer Research about a year ago concerning the activated macrophage. This is, again, not an acquired immune response but a macrophage that has the ability to kill tumor cells, to recognize them in some fashion and kill them while leaving the host cell alone. Immune surveillance is not a dead issue at the present time. Acquired immunity probably has more of a role once the tumor has progressed far enough so you start getting dead cells and maybe antigenicity at that particular point in time. Your point is very well taken that most of the metastatic tumors have weak antigenicity if any at all.

DR. LAWRENCE (Albany Medical College of Union University): Can I make one comment in reference to that statement? I think it should be pointed out as well that immune response is not necessarily always a positive response. If you're looking for a positive response it may not be there, but there may be a negative response. You may have induction of suppression which in fact enhances or aids the ability of the tumor to establish itself. You wouldn't really see it because you're usually looking for positive response.

DR. LUSTER (National Institute of Environmental Health Sciences): There is a large data base on individuals that are immunosuppressed for one reason or another whether caused by genetic immunodeficiency or by immunosupressive therapy on patients that have transplants. These individuals are very likely to have a much higher rate of neoplasia than the normal population. In that respect, Burnett's theory does apply to an extent.

DR. BAXTER (University of Cincinnati): I think Lloyd Old made a very good response to this question in his cancer research. I think it was at the Claus Memorial Award Lecture a couple of years ago where he said that what he thinks is happening is that the tumors are cunning enough to disguise themselves. That they may not, in fact, be able to fool the immune response. They may be putting out antigens to be recognized, but they may be disguised. What we have to do now is to find ways of making tumor cell antigens reveal their true selves. He stresses again that we're going through this state where the immune response, especially in tumor immunology, is just not well enough known at the present. We just don't realize the complexities, but when we do we'll have a better handle on the problem.

DR. KERKVLIET (Oregon State University): I think that the area of chemically induced effects can also get at the question of basic tumor immunity. We're not just looking at it from a toxicologic standpoint, but hope to be able to use these as tools to answer some questions about the basic role of the immune system. That's why I think it's also important to use tumor models in looking at the immune response in the mouse - not just to do the sheep red blood cell assay, the macrophage assay, or the in vivo tumor susceptibility assay and then try to paint a picture of what's happening. Although I didn't talk about it today, we have also used the Maloney sarcoma virus system to look at the immune responses going on in the mouse during various phases of tumor growth.

DR. AUST (Michigan State University): I would like to comment that quinone is redox active and to ask Dr. Pfeifer two questions. Are there enzymes in the cell that can catalyze reduction of quinone? Secondly, what is the possibility of metal catalyzed sulfhydryl-dependent reduction of quinone?

DR. PFEIFER (National Institute of Environmental Health Sciences): That's an interesting point. It's true that within the cell something like that may be going on but it's my belief that these sulfhydryl sites are being alkylated. Under the conditions of the experiment it certainly would appear to be an alkylation process

that's happening at physiologic pH when you follow the activity of hydroquinone as it interacts with protein. There is a possibility that what you suggest could be happening inside the cell.

DR. ALI (Ohio State University School of Medicine): I would like to ask Dr. Baxter about the inflammation that he saw after the topical application of phorbol on the mouse. How long after treatment do you see inflammation? My second question is about the tumor. Does the inflammation persist after the tumor develops?

DR. BAXTER (University of Cincinnati): In answer to the second question, by the time you have tumors the inflammation has subsided to grade one or two level. After about the third application the inflammation is at its peak which is about grade four. It's very severe. You get inflammation very rapidly from the first application. Usually within twenty-four hours after application of the phorbol ester inflammation starts to set in.

DR. ALI: The next question, can we exclude this effect of inflammation from being just a local effect due to irritation from the phorbol rather than an immune mediated effect?

DR. BAXTER: That isn't really known and I don't think enough work has been done in this system to make that statement. We're definitely showing systemic effects by topical promotion by which I mean that the topical administration of the TPA is causing systemic effects in the lymphoid tissues. Are you asking whether the infiltrating leukocytes arise locally or whether they come from the bone marrow?

DR. ALI: That is correct.

DR. BAXTER: We don't know. I don't think anyone has done enough work in this area yet to determine their origin.

DR. KAROL (University of Pittsburgh): I'd like to ask Dr. Baxter a question on that subject. Have you ever tried to reduce the inflammatory reaction after you administer the ester and see if you still get tumor promotion? For example, giving a steroid to reduce the inflammation.

DR. BAXTER (University of Cincinnati): Fluocinolone acetonide is just that kind of compound.

DR KAROL: I see, so you still get the effect.

DR. BAXTER: No, it's also a potent inhibitor of promotion which makes a strong case for inflammation and carcinogenesis in this tissue. As I said, inflammation has not yet been definitively ruled out as an important facet of carcinogenesis in this or really in any other model in the skin. Many induced and some spontaneously arising tumors are from macrophages. There is a heavy immune involvement which may be related to inflammation. Inflammation comes in many shapes and sizes. It appears that if you reduce inflammation, you also get reduction of promotion of skin tumors.

DR. HAFEMAN (Case Western Reserve University): I have a question for Dr. Pfeifer. What system did you use to measure tubulin polymerization? Was there a decreased rate of polymerization or an increased rate of depolymerization caused by hydroquinone?

DR. PFEIFER (National Institute of Environmental Health Sciences): It was a cycle purified rat brain tubulin incubated in vitro in phosphate buffer. Our measurements were made after a ten minute incubation and the values I reported were effects of the polymerization and not effects of depolymerization.

MR. VERNOT (University of California, Irvine): My question is for Dr. Kerkvliet. If I remember correctly, the slide you showed of the induction of splenic tumors indicated that the pure pentachlorophenol was very effective in inducing a high incidence of tumors, whereas it was almost totally ineffective in the induction of tumors at the site of PCP application. I wondered whether you had any explanation for this apparent difference?

DR. KERKVLIET (Oregon State University): At the present time, I don't really have an explanation. My hypothesis is that there is some immunosuppression associated with the pure product. I am assuming that the development of splenic tumors is due to immunosuppression. That is based on what has been shown in the literature from some of the more standardized tests done fifteen to twenty years ago with MSV. Right now, it's a fascinating thing that looks like it could be a very sensitive detector but we'd have to do a lot more work. It also would be a long-term assay because you want to wait on your secondary challenge to allow a real strong response to come down. It would probably be a six-month assay.

DR. IRONS (Chemical Industry Institute of Toxicology): I'd like to respond to the earlier question on the tubulin assembly assay. That was a kinetic analysis. We looked at assembly over a

ten-minute period and in that particular system it would appear that there is not an alteration in the initial rate of assembly but a decrease in maximum assembly. I think it's somewhat controversial because the whole area of relating the physical chemistry of GTP hydrolysis with tumor assembly is not fully accepted at this point.

DR. KLEMME (National Institute for Occupational Safety and Health): I have some questions for Dr. Karol. I think all three are really the same question, so let me ask all three at once. In your animal system, animals were exposed to TDI for seventy days at a low level and were reported not to have shown any immune response. Is there any reason to expect that they would not show response if they had been maintained at that level of exposure for a longer period? The second question, do you have data for mice that have been sensitized by higher levels of exposure to find out just how far down they remain sensitive in terms of how small a dose will still elicit a second response? Finally, is there a difference between animals that have never seen TDI before and ones that have been exposed at a low level that has not elicited a measureable response?

DR. KAROL (University of Pittsburgh): With regard to the first question, we exposed animals for seventy days in order to accumulate a total dose of TDI in terms of ppm-hours that gave maximum sensitivity in our other studies. I can't imagine why a response would develop after 140 days as opposed to 70 days since their total exposure was optimal in those 70 days. The accumulated dose was enough to produce an optimal response so that's why seventy days was picked. All of the experiments are done so far with guinea pigs because guinea pigs give you a very good pulmonary sensitivity reaction, mice don't so that all the tests or the animal models using small animals would be done with guinea pigs if you want to look at a lung sensitivity reaction. The third question, I can't remember.

DR. KLEMME: Maybe I didn't make the second question very clear. The second question I had intended to ask was about animals that have been exposed to higher levels of TDI and in which immune response has been elicited. How small a dose will still elicit a renewed response?

DR. KAROL: That's a very interesting question. We've done that with protein antigens. We've sensitized animals to small proteins and then challenged them and gotten very nice lung reactions and they had high antibody titers and then we let them rest for a couple of months, and then re-challenged them and they had exquisite sensitivity - very good lung reactions and they still had maintained some antibody level. That wasn't a full scale experiment, but that would be a very interesting question to see how the lung sensitivity

decreases over time and how the antibody titer decreases over time in certain defined systems. What I described was just a short rest period and the lung sensitivity was exquisite when we re-challenged.

DR. KLEMME: If you exposed those animals to high TDI levels after seventy days of very low levels and compared their response to animals that were seeing TDI for the very first time, would there be a difference in response?

DR. KAROL: I could only guess. We haven't done that experiment and my guess would be, no, there wouldn't be a difference because it looks as if that exposure for seventy days caused no recognition by the immune system at all. The immune system just couldn't respond to that type of exposure. So I would guess that they would respond as naive animals would.

DR. CROCKER (University of California, Irvine): Dr. Pfeifer, you mentioned briefly that tubulin integrity might be disturbed by autoxidative events. Is that correct?

DR. PFEIFER (National Institute of Environmental Health Sciences): During incubation under physiologic conditions, the hydroquinone is converted to a parabenzoquinone structure within minutes. It can be followed spectrophotometrically in that sort of buffered environment. We do the pre-incubation with cells without any calf serum in the media, just exposing the cells to the product of the hydroquinone reaction. The cells are really being exposed to parabenzoquinone.

DR. CROCKER: Could I just take that one step further. When such an effect has been produced, do the cells have tubulin destabilization and lose the rod shape of the structure?

DR. PFEIFER: I would say that the alkylation is on the six S units of the tubulin so we're really preventing tubulin polymerization from taking place. With PHA added to a culture, there is going to be an initiated stimulus to form tubulin polymers and for the elongation event to take place. Sulfhydryl alkylation blocks that series of events from taking place.

DR. CROCKER: Then in addition to the mitogenic effects, is there a loss of the integrity of the sheeted cells as a culture or as an adherent cell?

DR. PFEIFER: At those same concentrations, they completely lose adherence abilities. If you look at the membrane, they seem to be intact when you use trypan blue exclusion or if you measure the energy levels, they have normal amounts of ATP. There seems to be selective toxicity at micromolar concentrations of these agents. If you incubate with much higher concentrations, you might start seeing other toxic events.

DR. YAGER (University of California, Berkeley): Dr. Karol, this is for my elucidation, in the guinea pig study, you looked at three endpoints of sensitivity cytophilic antibodies which are IgE, pulmonary response, and dermal response. How would you categorize these last two endpoints in terms of the types of response being measured? How obtuse is dermal measurement of IgE?

DR. KAROL (University of Pittsburgh): In humans the immediate hypersensitivity response is due to IgE antibodies. In the guinea pig, for the most part, it's due to an IgG 1 type antibody. It has the same function as IgE when it binds to cells and it's cytophilic and it causes the release of mediators on contact with antigen, but it's IgG 1. We measured that type of antibody by doing a cytophilic assay in which we measured the total antibody that has the function of causing release of mediators in the guinea pig. The skin sensitivity we saw was delayed sensitivity to TDI. We were not measuring IgE antibodies because the sensitivity was delayed several hours. The lung sensitivity was an immediate response that came during challenge or within the immediate postchallenge period. The challenge period lasted about ten minutes. We were looking at three different indications of sensitivity, two of which measured immediate sensitivity and the skin was actually measuring a delayed reaction.

DR. YAGER: Was it a cellular response?

DR. KAROL: We would have to prove that it's cellular. I really don't know what mediated that skin reaction to TDI but we didn't see it within the first hour. It took up to 6 hours to arise.

DR. COURI (Ohio State University School of Medicine): Dr. Karol, I would like to ask a question about the RAST antibody titer in individuals who are ready reactors. Is that a good response or a bad response? In other words, does that protect that worker, or does that make him more susceptible to serious injury?

DR. KAROL (University of Pittsburgh): Usually an antibody response is protective. In the RAST though, we're measuring IgE and IgE is a harmful antibody. If a person has a high IgE titer, it's a warning that they have a hypersensitivity response because they have the appropriate antibody that could produce this hypersensitivity response. A high RAST is usually a warning that this person could undergo an asthmatic reaction.

DR. COURI: Since you're interested in re-challenge, what happens when they are re-challenged three months later?

DR. KAROL: One worker whom we followed for three years was re-challenged several years after his last exposure and he didn't respond to challenge. He was challenged with 0.02 ppm which is the normal workplace level and he was no longer sensitive to his environment. He did not react.

DR. COURI: May I ask a question in general to the panel? I appreciate what all of you have told us, but something occurs to me. Are there tools that are missing? I think we're looking at the effect of chemicals on the immune system by making a lot of cellular measurements or subcellular measurements, or treating in vivo and then measuring in vitro. Some of you have already suggested that what happens in vitro really never happens in vivo. What are the tools that are yet necessary to really get a little more definitive on what is stimulation to response or enhancement, and what is suppression of response? Is that too general?

DR. SONNENFELD (University of Louisville School of Medicine): Yes, you're very right. There were many tools missing in our interferon work. We couldn't even get a purified interferon molecule until this year and to get an antibody against pure interferon was very difficult. These tools are now available and will probably allow us to carry these studies much further. As far as the interferon system is concerned, there has been some very limited in vivo work, something that was done a long time ago with urethane, one paper from the German literature about 3-methylcholanthrene, some of the things we've done and some work done in Dr. Archer's laboratory that indicate that in vivo effects are similar to what happens in vitro. However, that still doesn't explain what role this might have in the actual development of a tumor. The probes are just now becoming available to do those kinds of studies.

DR. WHITTENBERGER (University of California, Irvine Southern Occupational Health Center): I've been wondering how sensitive the immune system is to environmental chemicals in comparison with other systems of the body and I've been wondering how realistic the

exposure levels are that you've all been talking about today. The only person that I heard address the second part of my question was Dr. Karol who developed an animal system and then tested it against a situation in the workplace where there was some very low concentration of TDI and showed the comparability between the respiratory response to TDI and the immunologic response. Perhaps Dr. Munson would like to answer the first part of my question?

DR. MUNSON (Medical College of Virginia): I'm not sure that this question is specific to immunotoxicolgy. What goes on in immunotoxicology is what goes on in toxicology. We try to demonstrate a dose response and we try to work in an area of the dose response curve where we can get a reproducibility response and then hopefully attempt to extrapolate it to realistic levels. I think we all know all the pitfalls involved in that. More specifically, I think that with some of the chemicals that have been discussed today, we are not very far away from realistic levels. The lowest level of diethylstilbestrol we talked about was 2 milligrams per kilogram which is still fairly high; we do know that this level will affect microsomal enzymes. Overall, when you look at some of the chemicals that have been found to have an effect on the immune system, they generally will work at relatively low levels and therefore, I would say that they're realistic studies. I don't like that argument because I still think we have to deal with dose response and extrapolations. The sensitivity of the assays is continuing to improve. I think that Dr. Archer did a very nice job of outlining a number of the assays. The National Toxicology Program is trying to do intra-laboratory validation of a number of these assays.

DR. WHITTENBERGER: That was the second part of my question. I have the impression that immunotoxicologic testing have been exploding somewhat as the short-term mutagenicity bioassays have been exploding for several years. It was a long time before anyone began attempting to standardize the tests and compare various tests and response to the same stimulus. Dr. Archer did mention the NTP protocol with respect to its inclusion of some of the tests that he talked about. It wasn't clear to me exactly what he meant by having it included in the NTP regime.

Dr. ARCHER (Federal Food and Drug Administration): I think Dr. Luster should address that since he is in charge of that program.

DR. LUSTER (National Institute of Environmental Health Sciences): The National Toxicology Program represents an inter-agency agreement among NIEHS, NCI, NCTR, and FDA. It is used primarily for toxicity testing and carcinogenicity testing so there would be a data base available for regulatory agencies to make regulations. We felt as immunologists that in earlier studies immunotoxicology did

suffer from a lack of validation. The data weren't consistent from one laboratory to another. Therefore, a few years ago we set up several workshops for panels of experts in immunology, pathology, and toxicology. The panel identified all the immunologic assays they felt would represent the state of the art in immunology testing. The NTP subsequently arranged with several contract laboratories to validate those methods. The question posed was could several laboratories using similar species of animals at a similar dosage level with similar lots of fetal calf serum and other reagents obtain similar results? The data have been coming in over the past year and a half and the answer is yes. The first chemical that was used for inter-laboratory validation was diethylstilbestrol. When we did mitogen assays which are probably the more variable assays from one laboratory to another, at the highest dosage level tested, the percent suppression from controls measured at all three laboratories doing the validation test were between 60 and 70% which I thought was amazingly similar. The host resistance assays are starting to come into play. Assays that cannot be validated, in the sense that consistent results can't be obtained in our laboratory, are not candidates for testing in the contract laboratories. We are trying to make or find assays that are representative.

DR. LAWRENCE (Albany Medical College of Union University): I'd like to address a question that came up earlier. I may have fostered some of the confusion in terms of differences between in vitro and in vivo testing. It's very necessary to look at the in vitro situation if you want to clearly define the immune system and the specific function of its components such as T cells, B cells, macrophages, and others. You're not looking at the same situation in vivo. You have many secondary effects that are taking place in vivo with lymphocytes, antigens, accessory cell functions on T cells, B cells and macrophages. There may be differences due to factors from other cell types in the area and there may be micro-environmental differences. You really have to study both systems, in vitro and in vivo, if you want to not only get a feel for whether there is immuno suppression or immuno enhancement but if you want to assess whether that effect is due to B cell, T cell, or macrophage reactivity. They don't necessarily correlate. If they do not, then you know that there may be secondary parameters that need to be looked at.

DR. IRONS (Chemical Industry Institute of Toxicology): I'd like to expand on Dr. Luster's comments about interlaboratory validation. It is true that currently we are doing this under contract with NIEHS, using DES as one of the compounds and looking at trichonella for example. However, I'd like to point out that this type of interlaboratory validation had been going on for a number of years before NTP sponsorship with host resistance assays. Both CIIT and the EPA have been using the streptococcal model for six or perhaps seven years, looking at effects of nitrogen dioxide and ozone. There's been excellent agreement with these host systems.

MR. TOBIA (Ohio State University School of Medicine): Dr. Lawrence, I'd like to know why you didn't study gold? At least I didn't see that on the table you presented. You did a lot of good work with lead but since gold is now a very hot topic in immunology I'd like to know why you didn't study it.

DR. LAWRENCE (Albany Medical College of Union University): Because it was too expensive. We're doing studies with gold right now because of its effects on rheumatoid arthritis. The studies are being done on the function of gold in regulating immune regulation, and suppressor T cell and helper T cell activities. We're beginning to look at gold, but it wasn't on our list initially because it cost too much!

DR. AUST (Michigan State University): I think we may be making the field a little more confusing than we need to right now at this moment. There are people here that are interested in the immune system. There are attendees who are interested in the mechanism of toxicity and others who have to set regulations and I think we cause confusion by asking questions like, "Well, did you include one of the tests that NTP has decided is a critical test in your immunology study?" If you're interested in setting TLV's, you may answer yes, but if you're studying the mechanisms of immune system functions, you may say no and I have no interest in doing such. There are researchers who use chemicals to perturb the immune system that just happen to be something that contaminates our environment or are used in the workplace. The question of whether the chemical is safe or unsafe may be related to a TLV but that question should only be asked when someone is actually trying to set a TLV and not when that person is interested in a mechanism by which a chemical affects the immune system. I would like to note that I did not say how a chemical is immunotoxic. I think there is a big difference between studying immunotoxicity and studying the effect of chemicals on the immune system.

DR. WHITTENBERGER (University of California, Irvine Southern Occupational Health Center): They are different objectives, but they're both important.

DR. LAST (University of California, Davis): I would like to expand a bit further on what Dr. Aust said because I think it is an important point. A lot of the assays that were discussed today are based on infecting animals with very large innocula of bacteria, of viruses, or of tumor cells. This is done for experimental convenience if you want to determine LD_{50}'s or if you want to palpate a tumor in a short period of time. The response of the host to this kind of overwhelming innoculum may be very different from the normal response of a host who initially is exposed to a level several log

doses lower that may replicate in the host. In that case, it may again be misleading to talk about the exact dosage of the chemical that is required to modify this response or to talk about detailed mechanisms that may be occurring on an immunologic basis in these animals as if they mimicked the in vivo response to a xenobiotic in a normal host. I think we have to also distinguish what we do for our own experimental convenience from that which accurately replicates normal processes that occur at low levels of infection as well as at low levels of chemical exposure.

Dr. ARCHER (Federal Food and Drug Administration): I'd like to make a comment, both on Dr. Aust and Dr. Last's statements about in vitro assays. They interrelate in a way. Dr. Aust more or less put immunotoxicology in a box by itself. I think there are more than two ways to look at anything. The two ways I see that you can look at immunology or the immune system, first, for the sake of finding out if a chemical is a hazard to the immune system and as such a hazard to the host and the other way of looking at the immune system is as a model in general biochemical toxicology. Cells are cells and lymphocytes happen to be very specialized cells but there is an awful lot known about them that make them a very valuable tool. Now this is where it gets into the in vitro situation that was asked on a previous question. Just to give you an example that I am familiar with, our original in vitro work with food-borne antioxidants predicted that BHA was an immunosuppressive chemical. When we studied the in vivo situation, that concept turned out to be totally untrue. Possibly because of distribution of metabolism factors, possibly other reasons, I don't know. In vitro, however, it's a very potent immunosuppressant. Later, using in vitro systems we learned that the cyclic nucleotide systems were involved on a cellular basis. In whole animal studies, we learned that BHA is a protective chemical in some instances against a whole variety of carcinogenic materials. Dr. Lee Wattenberg at the University of Minnesota has shown BHA to be an antipromoter agent on epidermal cells and that the only organs in an in vivo situation that are protected by BHA are cells with which it comes in direct contact. So in that situation, the in vitro data that we had collected may have been a mirror image of what BHA could do to cells with which it came into direct contact, so in that sense the in vitro systems were valuable. But in the other context of Dr. Aust's question, that's a whole other realm of what immunology can do in the context of toxicology. That is, it can be a very, very exquisitely sensitive and very easily dissectible way of looking at general biochemical toxicologic phenomena.

SESSION III

COMPARATIVE RESPONSES TO INHALED TOXICANTS

Chairman

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PAPER 11

UPTAKE AND FATE OF INHALED PARTICLES AND GASES: THE IMPORTANCE OF SPECIES DIFFERENCES

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INTRODUCTION

Dr. Whittenberger may be right that comparative physiology has been around for a long time, but the challenge of describing how different species respond to inhaled toxic agents is not a trivial task. Rather, we have been asked to discuss a central problem in toxicology: How do we interpret animal experiments to better predict the potential of an agent to cause damage in humans? In other words, how do we extrapolate from one species to another and from animals to humans?

Although many species have been used to assess the toxicity of chemicals, and although species differences are both recognized and argued about, we lack a complete and systematic description of the differences among commonly used laboratory animals. The subspecialty of inhalation toxicology is no exception. It is difficult to abstract a comprehensive description of species differences from the literature because so many different kinds of animals and aerosols have been used in various combinations. Several theoretical and experimental contributions exist, but the problem is far from solved. At least three aspects of exposure to toxic particles and gases should be considered. They are: deposition, clearance, and the magnitude and type of biological response.

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DEPOSITION

Palm and co-workers (1956) studied retention of dust in the lungs of guinea pigs and small monkeys; the percentage of alveolar retention as a function of particle size was substantially the same as that found earlier in humans. Friedlander (1964) used dimensional analysis to investigate deposition of particles in the lower lung. Kliment (1973) identified several of the important dimensionless groups of physiologic variables that appear in aerosol deposition problems and predicted the degree to which these groups of variables are the same in rats, guinea pigs, rabbits, and humans. More recently, Stauffer (1975) has used dimensional analysis to predict that the probability of deposition of inhaled aerosols should be the same for different animals in the case of sedimentation or turbulence-dominated deposition. The predictions of McMahon et al. (1977) differ from those of Stauffer (1975), and suggest that diffusion-dominated deposition is also independent of body weight.

McMahon et al. (1977) attempted to specify each of the physical mechanisms of particle deposition by identifying the controlling dimensionless group. For example, in the particle impaction problem, the collection efficiency, E (the percentage of particles entering that is actually deposited), increases as the Stokes' number, St, increases. The Stokes' number is the ratio of the stop distance of a particle to the characteristic dimension of the system. Similarly, the Froude Number, y, which is the ratio of the particle sedimentation velocity and the through-flow velocity, governs the sedimentation of particles in the nose, pharynx, and large airways during quiet breathing. Diffusion of small particles to the walls of the alveoli increases when the dimensionless diffusion time, DT/a^2, increases. D is the diffusion coefficient (which depends directly on temperature and inversely on air viscosity and particle radius), T is the breath period, and a is the alveolar diameter.

If all of the dimensionless groups varied with body weight, understanding the scaling rules for deposition of particles in the lungs would be complicated and difficult. Fortunately, several of the dimensionless groups are independent of body weight. Although many important physiologic parameters, such as breath period and ventilation, change as body weight changes, competing effects sometimes cancel one another in the controlling dimensionless groups. Then the collection efficiency is independent of body size for the same aerosol size.

McMahon and co-workers (1977) tested these ideas with a "Noah's Ark" approach. They simultaneously exposed 6 different species to the same 0.78μm aerosol of gold-198 and compared both total deposition and site of deposition among species. When the total amount of aerosol deposited was divided by the animal's

body weight, it was found that the smaller animals received more particles/gm than the larger ones. However, as expected, the collection efficiencies (the fraction of inhaled aerosol deposited) for both the lungs alone and the lung, nose, pharynx, and airways combined were substantially independent of body size.

So far, we have discussed deposition in relation to normal breathing patterns at rest. I now want to remind you that many of the things we do as toxicologists will affect the breathing pattern. The breathing pattern in turn will affect the dose of particles or gases retained by the animal. When you restrain an animal and give a head-only exposure, the breathing pattern changes. Generally, the animal will breathe faster and more shallowly. Exposures to irritant gases, such as ozone, will do the same thing; they also generally result in a shallower and more rapid ventilation. These changes in breathing pattern can have a profound effect on the lung dose.

Recently, Valberg et al. (1982) have described how breathing pattern can influence deposition sites of an inhaled ^{99m}Tc-labeled aerosol. First, we found that the collection efficiency varied from 2% to 40% as we explored a variety of breathing patterns. Not only did the collection efficiency (the fraction of inhaled aerosol deposited) change, but there were also significant changes in the distribution of retained aerosol. After the aerosol exposure the lungs were inflated, dried in a microwave oven, and sliced (see Figure 1).

Figure 1. A slice of dog lung exposed to a submicronic ^{99m}Tc-labeled aerosol. After aerosol exposure, the lungs were fully inflated to 35 cm H_2O pressure and dried in a microwave oven. The rigid lungs were then sliced at 1 cm intervals.

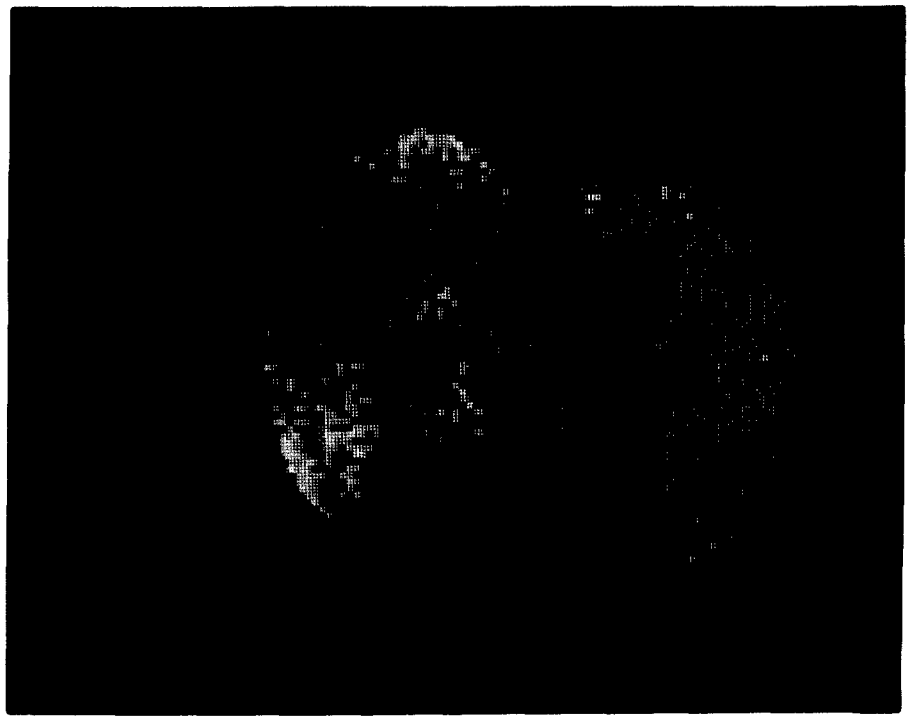

Figure 2. Slices prepared as described in Figure 1 were placed on a gamma camera with a high-resolution collimator and interfaced with a computer. Shown here is a photograph of the cathode ray tube out-put of the gamma camera showing the distribution of radioactivity over the slices of dried dog lungs. Each picture is a matrix of 128 x 128 cells that shows the activity with white and orange as high activity and blue and black as low and no activity. The lungs shown here were ventilated with a large tidal volume and a low breathing frequency. Note that the aerosol distribution through the parenchyma is relatively uniform but the airways are relatively free of retained particles.

You can easily identify the pleural surface, pulmonary parenchyma, and large and small airways. Then we used a number of techniques to look at the distribution of retained aerosol in such a slice. You can take this slice and lay it on the face of a gamma camera. With a slow deep pattern of breathing, the amount of aerosol deposited in the airways is relatively less than that in the parenchyma (Figure 2). With a rapid, shallow breathing pattern, there is much more deposition in airways than there is in the cooler parenchyma (Figure 3).

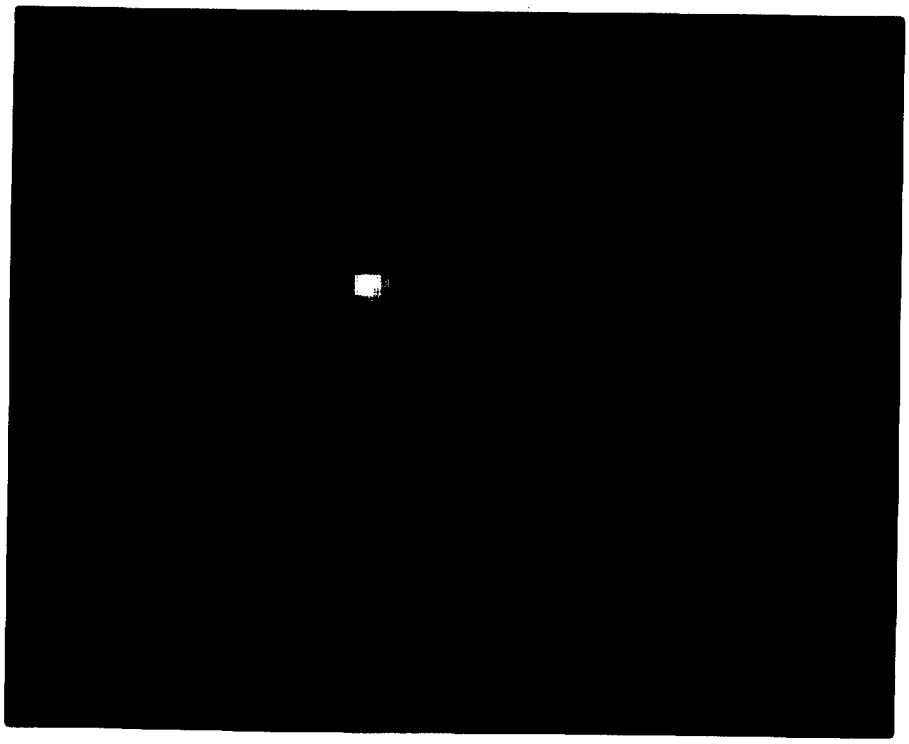

Figure 3. The slices shown here were prepared as described in Figures 1 and 2, but the lungs were ventilated with a small tidal volume and at a high breathing frequency. Even though the sub-micronic aerosol used was the same in both instances, now the retention pattern is completely reversed. Airways now contain more radioactive particles than the "cooler" parenchyma. Comparisons to photographs of the same lung (similar to Figure 1) clearly show that the red-orange-white areas correspond to airways.

The computer attached to the gamma camera translates the amount of activity in a region to color. The colors get warmer (red, orange, orange, white) as we get more and more activity. It is blue or black if there is very little or no activity. The same patterns of retained particles can be demonstrated with autoradiographs or by counting and weighing individual pieces from the lung slices (Valberg et al., 1982). These changes in retention pattern caused by breathing pattern will, over time, have a profound effect on the dose. Material deposited in airways will be cleared more quickly than particles deposited in the parenchyma.

CLEARANCE

It is essential to realize that different rates of clearance among species can also influence retention and, therefore, the total dose to the lung. All aspects of clearance may differ. We have recently examined mouse, hamster, rat, and rabbit in regard to the rate at which insoluble gold particles are taken up by pulmonary macrophages in situ (J. Brain, personal observation). Hamsters had the shortest half-time, followed by rats, rabbits, and mice. The observed in vivo differences were not completely accounted for by different in vitro activities of the macrophages or by different sizes or numbers of macrophages in the respiratory tract.

When the particles ingested by pulmonary macrophages are viable organisms, the efficiency with which the organisms are inactivated becomes a crucial factor in lung defense. The ability of the pathogen to cause lung damage and disease is in direct competition with the host's ability to mount an effective inactivation process. Different species demonstrate varying degrees of efficiency in this defense mechanism. When pulmonary antimicrobial defenses are examined, they also show marked species differences in the bactericidal activity of alveolar macrophages. For example, rabbit alveolar macrophages avidly ingest and kill Staphylococcus aureus, whereas alveolar macrophages from rats ingest but are rather poor at killing the same organisms. Also, Nguyen et al. (1982) reported significant differences in phagocytosis and killing by pulmonary macrophages from humans, rabbits, rats, and hamsters. In the absence of serum opsonins, macrophages recovered from humans were able to phagocytize Staphylococcus aureus, Cowan I (protein A positive). In contrast, pulmonary macrophages lavaged from rabbit, rat, and hamster, did not phagocytize Staphylococcus aureus, Cowan I, or other nonopsonized bacteria in the test system studied.

Felicetti and associates (1981) have shown that tracheal mucous velocity varies with species and is best correlated with tracheal surface area $^{0.57}$. They found that the clearance of intratracheally instilled ^{99}MTc-macroaggregates of albumin was found to be faster and more efficient in dogs than in smaller animals. If one includes non-mammalian species, even greater variability can be encountered. In boa constrictors, Grant et al. (1981) demonstrated that 41% of an inhaled radiolabeled submicrometric aerosol retained 5 hours after the end of the exposure was found in the trachea. This suggests a much slower mucociliary transport system than has been observed in mammals. Interestingly, Grant et al. (1981) also reported that less than 10% of the snake tracheal epithelium was ciliated. While studying chickens, Mensah and Brain (In press) showed that lung clearance of submicrometric particles has a biphasic pattern: a fast

phase, where clearance is faster than has been observed for hamsters or mice; and a slower phase. Also, Thomas (1972) has developed a model describing the kinetics of clearance of inhaled particles in the respiratory tract of mice, rats, and dogs.

Together, the relative rates of deposition and clearance determine the amount of a substance present in the respiratory tract; this is called the retention. If exposure is continuous, then the equilibrium concentration (achieved when the clearance rate matches the deposition rate), is also the retention. It is the retention integrated over time as well as the metabolism and properties of the particles that are presumably related to the magnitude of the toxic reponse.

In conclusion, it is important to remember that the species selected for exposure will influence the resulting dose to the lungs. Different species breathing the same aerosol do not receive identical doses. Exposure concentration (e.g., "mg/m^3" or "ppm") is not an adequate description of lung dose. Even when the same atmosphere is breathed by different species, very different amounts and distribution of retained particles may still result. There are both systematic and unusual variations in ventilation, collection efficiency, lung anatomy, and clearance mechanisms among species, which influence the local doses of retained aerosols and gases.

BIOLOGICAL RESPONSE

Even if different species had the same lung dose of a toxic particle or chemical, it is still unlikely that the extent of lung damage would be identical. Besides variations in deposition and clearance, varying responses also reflect differences in the activation, degradation, excretion, or mechanism of action of the compound in each species. The innate responsiveness of the analogous cell, tissue, or organ may also vary among species.

An example is the case of the anti-tuberculosis drug Isoniazid which is well tolerated at doses of 100 mg/kg in monkeys, whereas in dogs, doses of 20 mg/kg produce convulsion, respiratory failure, and death. It has been found that monkeys almost completely acetylate (thus inactivate) INH, while dogs cannot (Coulston, 1966).

The toxic effect of prolonged exposure to 100% O_2 has been known for a long time (Bean, 1945). Lung damage includes interstitial and alveolar edema, and progressive respiratory distress, sometimes leading to death (Crapo and Tierney, 1974). Among the mechanisms proposed for lung toxicity are the formation of free radicals, chain reactions, and destructive oxidations (Gerschman, 1964). One important free radical is the superoxide anion; the

development of tolerance to oxygen toxicity may involve the ability to dismutate the anion via an increase in the activity of pulmonary superoxide dismutase (SOD). Crapo and Tierney (1974) have shown that the rate of development of O_2 tolerance closely parallels the time-course for the increase in pulmonary SOD activity in the rat. However, in guinea pigs, hamsters, and mice who do not develop tolerance under similar circumstances as the rat, there was not as large an increase in SOD activity.

Frequently, different responses to toxic agents reflect anatomical differences. For example, there are significant interspecies differences in the fine structure of the respiratory tract which can result in differences in the degree and distribution of damage after exposure to harmful aerosols or gases. Several studies have shown that rats develop lesions in terminal bronchioles and alveoli of proximal alveolar ducts following exposure to ozone (Castleman et al., 1973), whereas in monkeys, the major focus of damage is the respiratory bronchiole (Dungworth et al., 1975). The differences in the distribution of damage may be explained in part by the structure of the respiratory tract epithelia. Respiratory bronchioles are well developed in Macaque monkeys and are lined by non-ciliated epithelium comprised of cuboidal cells interspersed among squamous cells (Castleman et al., 1979). Non-respiratory bronchioles are greatly abbreviated. Rats, on the other hand, do not have well-developed respiratory bronchioles; the pulmonary acinus is simpler, and the terminal bronchioles open into alveolar ducts.

A similar structural difference has been proposed as the basis for the resistance of avian lungs to inhaled O_2 at 1 ATA. When chickens and rabbits are exposed to 100% O_2, the rabbits die within 5 days, while the chickens remain unaffected at 19 days (Somayajulu et al., 1978). Oxidant-sensitive ciliated cells are found in the respiratory airways of rabbits but not in chickens, and this difference may well underlie the difference in response to hyperoxia observed in the two species (Somayajulu et al., 1978).

Different species breathing the same aerosol do not receive identical doses. Exposure concentration (e.g., "mg/m^3" or "ppm") is not an adequate description of lung dose. Even when the same atmosphere is breathed by different species, very different lung doses may still result. There are both systematic and unusual variations in ventilation, collection efficiency, lung anatomy, and clearance mechanisms among species which influence the amount and distribution of retained aerosols and gases. Many other differences in reponses exist among species, but all too frequently we lack even hypotheses for their existence. A comprehensive view of species differences, with predictive power, is lacking but remains a worthy goal.

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PAPER NO. 12

COMPARISONS OF THE RESPIRATORY FUNCTIONAL RESPONSES OF ANIMALS AND MAN*

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INTRODUCTION

Respiratory function tests are widely applied to man as diagnostic tools, for occupational screening and monitoring, in epidemiologic surveys, to determine impairment for disability compensation, and as tools for studying lung physiology. The utility of these assays lies in their ability to provide, by rapid and non-invasive means, quantitative information about the functional status of the respiratory system. Although these tests address functional rather than anatomic realities. recognizable patterns of functional alterations are often pathognomonic for specific types of diseases. There is a strong correlation between measured functional abnormalities and subjective functional impairment in man, and the tests are useful in determining the extent of an individual's disability in relation to the requirements of his occupation (Morgan, 1982).

The development of methods for measuring the respiratory function of animals has roughly paralleled progress in human function testing (Likens and Mauderly, 1979). The first reports of simple measurements of breathing patterns of animals appeared in the early 1940s and applications of these assays in studies of the effects of toxic materials began in the 1950s. Considerable development in the ability to accurately perform sensitive, comprehensive measurements of the respiratory function of small laboratory animals such as rats and mice has occurred since the mid-1970s, and at present essentially all tests applied to man can be applied to experimental animals. The greatest impetus for this development has arisen from

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needs associated with toxicology studies, and the majority of work in the field today is related to toxicology. Therefore, information on the respiratory functional responses of animals to different forms of lung injury is rapidly accumulating.

The purpose of this report is to compare the respiratory functional responses of animals and man to similar, representative types of acute, subacute and chronic lung injuries. Examples have been selected in which human and animal lungs have been injured similarly, although in some cases the etiologic agents were different and in nearly all cases the doses and dosing patterns differed. The comparisons are intended to be largely qualitative; thus, detailed descriptions of dose-response differences, testing methodology, mechanisms of response, and underlying morphologic alterations are beyond the scope of this report. For further simplification, only comparisons using data from animals measured at this Institute are included. Definitions of symbols used in this report are listed in Table 1.

TABLE 1. DEFINITIONS OF SYMBOLS USED IN THE TEXT AND FIGURES

C_{dyn}	-	Dynamic lung compliance
C_L	-	Static or quasistatic lung compliance
DLCO	-	CO diffusing capacity
ERV	-	Expiratory reserve volume
f	-	Respiratory frequency
$FEF_{25,50}$	-	Forced expiratory flowrate at 25 or 50% of FVC
$FEV_{0-2,1}$	-	Forced expiratory volume at 0.2 or 1 second
FRC	-	Functional residual capacity
FVC	-	Forced vital capacity
IC	-	Inspiratory capacity
$P_{A-a}O_2$	-	Aleolar-arterial PO_2 difference
R_L	-	Airway resistance (total pulmonary resistance)
RV	-	Residual volume
TLC	-	Total lung capacity
VC	-	Vital capacity
V_D	-	Physiologic deadspace
V_E	-	Expired minute volume
V_T	-	Tidal volume

COMPARISONS OF FUNCTIONAL RESPONSES

ACUTE RESPONSES

Inhaled irritant aerosol - sulfuric acid (H_2SO_4)

An important respiratory functional response to inhaled gaseous or particulate irritants is airway constriction which occurs rapidly

and disappears after termination of exposure. The constriction is manifested subjectively as dyspnea and functionally as an altered breathing pattern and increased R_L. Concentrations of suspended sulfates have been correlated with asthmatic attacks in man during pollutant episodes (Cohen et al., 1972), and H_2SO_4 mist has been shown to be an airway constrictor in both man and animals.

Relatively high concentrations of H_2SO_4 are necessary to produce functional changes in man. Exposures to 1.0 mg/m^3 at various particle sizes have produced no significant functional changes in normal and asthmatic humans (Lawther, 1963; Sackner et al., 1978). Amdur et al. (1952) found that some human subjects had altered breathing patterns when exposed to 5 mg/m^3 of 1.0 µm H_2SO_4 for 15 minutes. Increases in R_L were measured by Sim and Pattle (1957) in healthy men who had inhaled 3-39 ($\bar{x}$ = 21) mg/m^3 of 1.5 µm H_2SO_4 for 30 minutes. The exposures caused intense lacrimation, rhinorrhea and coughing; thus the measurements could not be performed until 10 minutes after the termination of exposure. At that time, coughing was reduced and the measured R_L was increased to 143-250% of normal values (Figure 1).

Silbaugh et al. (1981a) exposed Hartley guinea pigs for 1 hour to 1-48 mg/m^3 of 1.0 µm H_2SO_4 and measured breathing patterns, C_{dyn}, and R_L during exposure. A portion of the animals inhaling 15-48 ($\bar{x}$ = 40) mg/m^3 developed dyspnea and had significant reductions of C_{dyn} and increases of R_L; the remainder were unresponsive. The R_L of the responders (10% responders at 15, 33% at 24 and 50% at 48 mg/m^3) increased to a mean of 390% of their individual baseline values (Figure 1) after 30 minutes of exposure. Thus, guinea pigs and men respond similarly to inhaled H_2SO_4.

There are significant interspecies differences among animals in their sensitivities to inhaled H_2SO_4. Wolff et al. (1979) exposed Hartley guinea pigs to various concentrations of 0.8 µm MMAD H_2SO_4 for 8 hours. Mortality during the exposure was related to the H_2SO_4 concentration (Figure 2) and animals that died first appeared dyspneic as did those described above in which functions was found to be altered. Histologic alterations were limited to the lungs and were consistent with airway constriction and local tissue hypoxia (Brownstein, 1980). Runkle and Hahn (1977) exposed CD-1 mice and Fischer 344 rats to various concentrations of H_2SO_4 and studied mortality and histopathology. Much higher concentrations were required to induce mortality than in guinea pigs (Figure 2). Histologic changes, particularly in the rats, suggested that death resulted more from laryngeal necrosis than from airway constriction. It is interesting to note that no mortality resulted from a later exposure of 8 rats to 1080 mg/m^3 H_2SO_4.

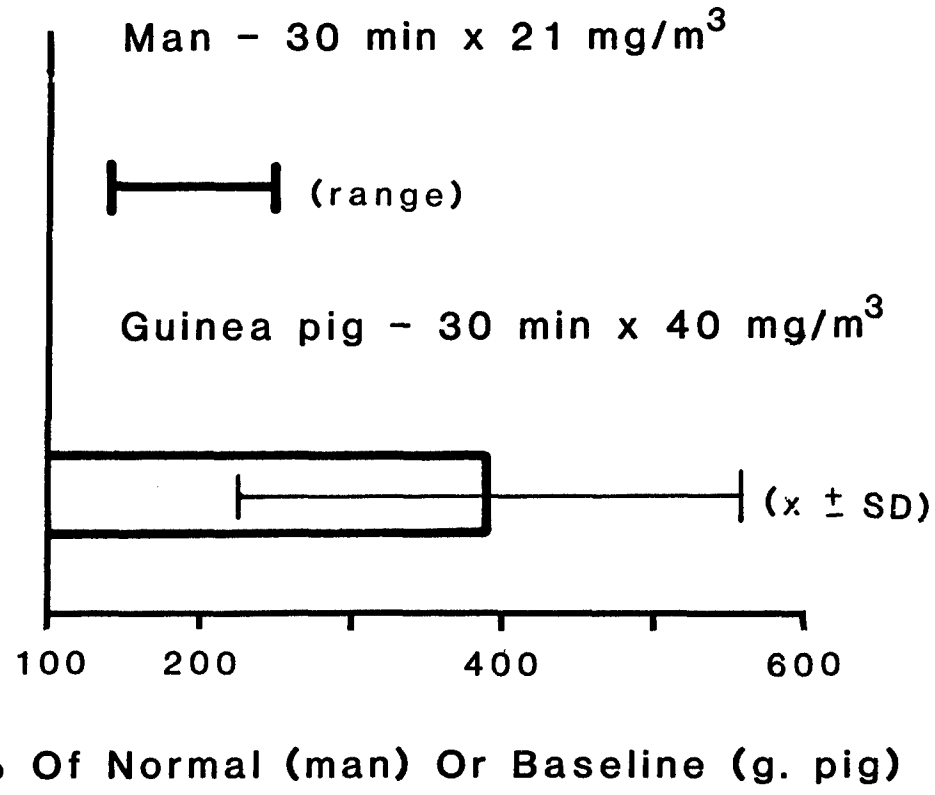

Figure 1. Effect of H_2SO_4 on Airway Resistance.

The increase in R_L of men (Sim and Pattle, 1957) and guinea pigs (Silbaugh et al., 1981a) after inhalation of H_2SO_4 for 30 minutes is shown. Values are expressed as the range of percent of normal for man and the mean ± SD percent of baseline R_L for guinea pigs.

These results demonstrate that guinea pigs exhibit both qualitative and quantitative functional responses to inhaled sulfuric acid much more similar to those of man than do rats or mice.

Effect of oxidant inhalation on airway reactivity

The inhalation of oxidant gases such as ozone (O_3) and nitrogen dioxide (NO_2) results in a transient increase in the sensitivity of human airways to the bronchoconstrictive effects of subsequently inhaled agents such as histamine and methacholine. Similarly, Silbaugh et al. (1981b) found that exposure to NO_2 temporarily increases the sensitivity of Hartley guinea pigs to histamine aerosol.

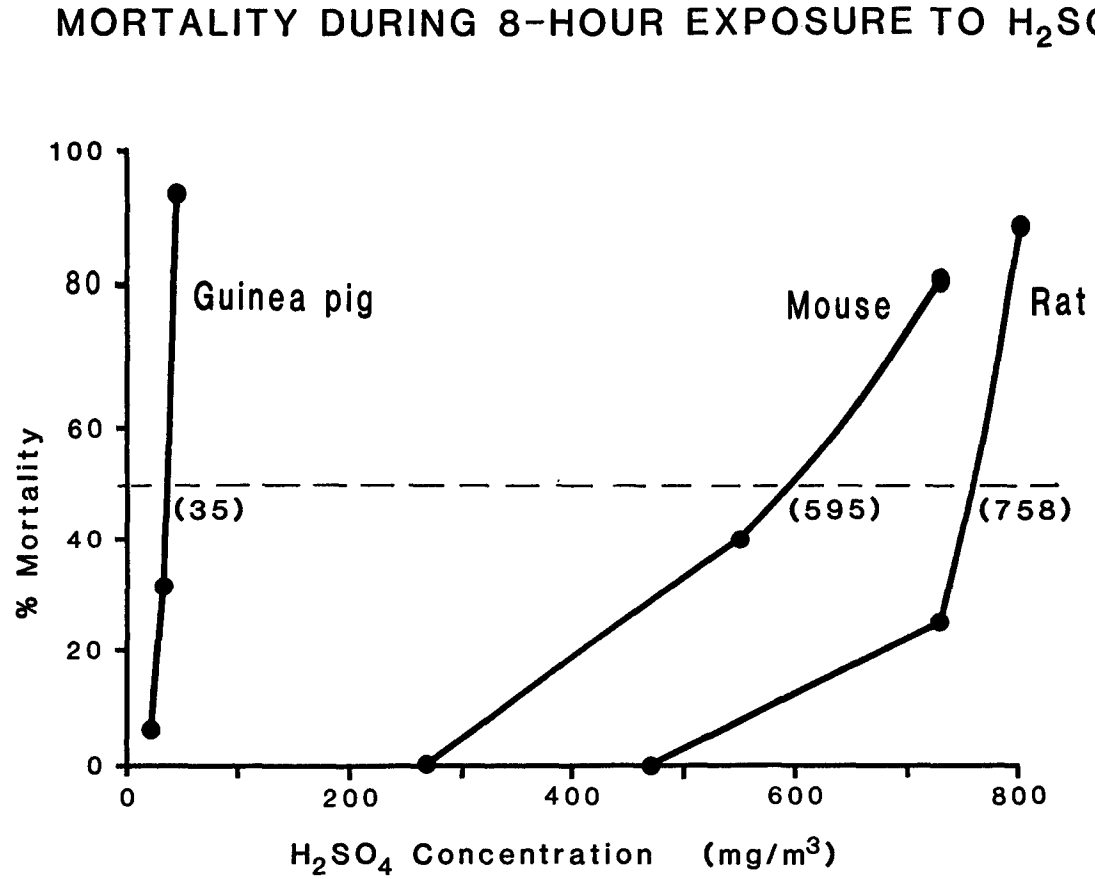

Figure 2. Mortality During 8-Hour Exposure to H_2SO_4.

The percentage mortality of Hartley guinea pigs (Wolff et al., 1979), CD-1 mice and F-344 rats (Runkle and Hahn, 1977) during an 8-hour inhalation exposure to various concentrations of 0.8-1.0 µm diameter H_2SO_4 aerosol is shown. Values in parentheses are the H_2SO_4 concentrations at which the straight-line plots intersect 50% mortality.

The responses of guinea pigs were compared to those of humans exposed to an oxidant gas and subsequently to histamine aerosol (Figure 3). Holtzman et al. (1979) measured specific R_L (R_L x thoracic gas volume) during histamine challenge before and at 1 and 24 hours after exposure of 9 atopic and 7 nonatopic, healthy human subjects to 0.6 ppm of O_3 for 2 hours. Histamine challenge was done by having the subjects take a standardized number of breaths of a constant concentration of histamine aerosol. For ease of comparison, specific airway conductance (the reciprocal of R_L) was plotted as a percentage of pre-O_3 exposure values in Figure 3. The mean conductance during challenge was reduced to 60% of baseline at 1 hour, but had returned to 93% of baseline at 24 hours after O_3 exposure.

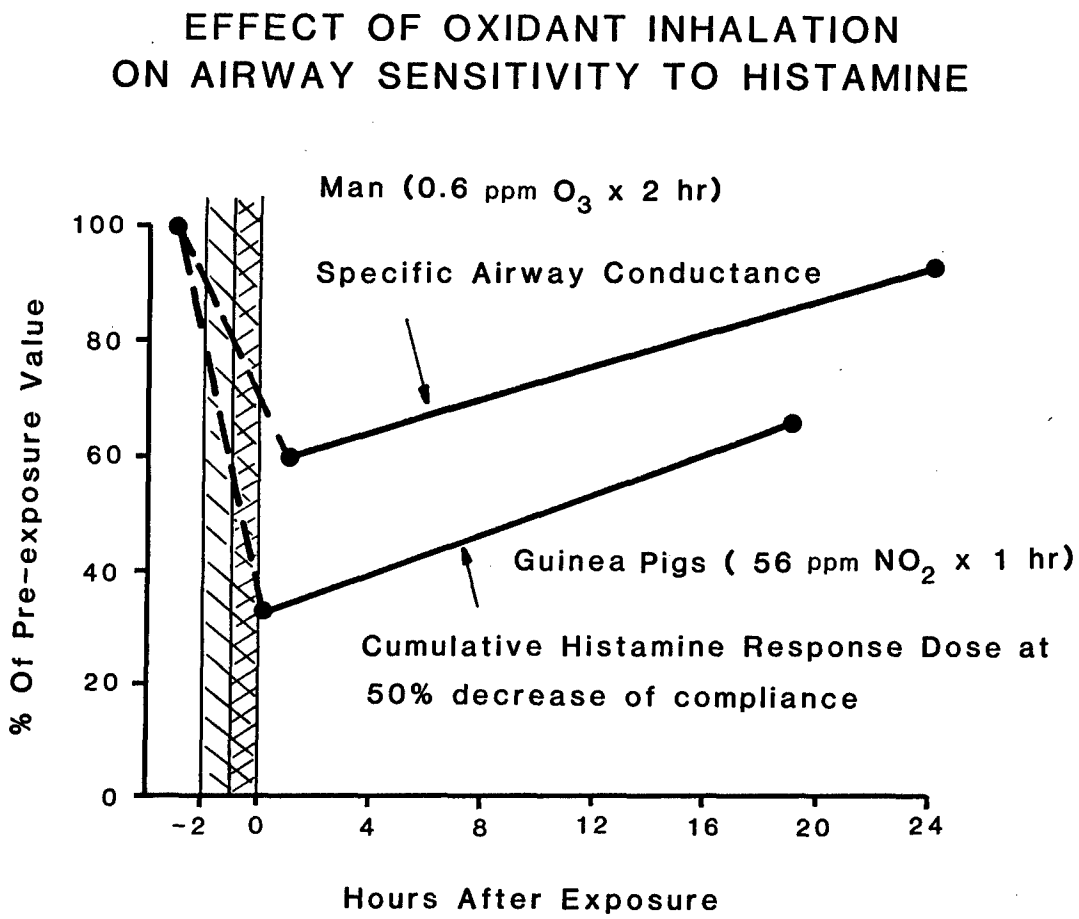

Figure 3. Effect of Oxidant Inhalation on Airway Sensitivity to Histamine.

The effect of oxidant inhalation on airway sensitivity of man (Hotzman et al., 1979) and guinea pigs (Silbaugh et al., 1981b) to subsequently inhaled histamine aerosol is shown. Cross-hatched areas indicate 2 hour exposures of man to O_3 and 1 hour exposures of guinea pigs to NO_2. Values are expressed as percentages of pre-oxidant exposure values. Transient increases in response to histamine are indicated by reductions in specific airway conductance of man and the cumulative histamine response dose of guinea pigs.

Silbaugh et al. (1981b) exposed 13 guinea pigs to 7-149 ($\bar{x}$ = 56) ppm of NO_2 for 1 hour and then measured C_{dyn} and R_L during histamine challenge at 10 minutes and 19 hours after NO_2 exposure. Histamine aerosol was delivered at stepwise increasing concentrations until a 50% reduction in C_{dyn} was achieved and the cumulative histamine response dose to reach that endpoint was

calculated. The mean cumulative histamine response dose was reduced to 33% of baseline at 10 minutes but had doubled to 66% of baseline at 19 hours after NO_2 exposure (Figure 3).

The comparison of Figure 3 illustrates qualitatively similar temporary sensitizations of human and guinea pig airways to the bronchoconstrictive effects of inhaled histamine after oxidant gas exposure. Although these data do not permit a quantitative comparison, the similarity of responses is obvious. It is particularly appropriate that both atopic and nonatopic human subjects were included in the comparison. Data from both the guinea pigs in this comparison and those discussed above (H_2SO_4 exposure) demonstrated wide ranges in airway sensitivity and the probable existence of "responsive" and "non-responsive" subpopulations. Thus, guinea pigs model man both in the existence of sensitization by oxidant gases and in the variability within healthy, adult populations.

SUBACUTE RESPONSES

Type III hypersensitivity pneumonitis

Type III hypersensitivity pneumonitis, a form of extrinsic allergic alveolitis, is an immunologic response of the lung to inhaled antigens which occurs in subjects previously sensitized to the agent. Many organic antigens have been implicated as etiologic agents (Richerson, 1980). In contrast to an immediate response, this disease is manifested by an inflammatory interstitial response which becomes apparent hours or days after exposure to the antigen. Morphologic changes in the more acute phases (as described in this comparison) comprise an interstitial granulomatous pneumonitis. Severe or repeated episodes lead to a chronic interstitial fibrosis.

The respiratory functional changes in humans with the acute form of hypersensitivity pneumonitis can vary with severity, but a good general description was given by Schlueter (1974). The subject becomes dyspneic hours after exposure; thus, the breathing pattern is altered. The VC and both C_L and C_{dyn} are reduced. Forced expiratory flow rates are reduced, as is the FEV_1; however, the FEV_1/FVC is usually unchanged. Gas exchange is impaired.

Likens et al. (1979) induced a hypersensitivity pneumonitis response in Fischer 344 rats that clearly resembled the response in man. Seven 15 week old male rats were sensitized by an intraperitoneal injection of ovalbumin given with killed Bordatella pertussis as an adjuvant and boosted with a second injection of ovalbumin 26 days later. Four days after boosting, the rats underwent respiratory function testing, were challenged with intratracheally instilled ovalbumin in saline and were retested 15 minutes later. No significant differences in function were detected at that time; however, the rats later became dyspneic and were retested 2-3 days

after instillation of ovalbumin. Marked changes in function were present at that time (Figure 4). The breathing pattern was altered toward a higher f and $\dot{V}_E$ and reduced V_T (Figure 4-A).

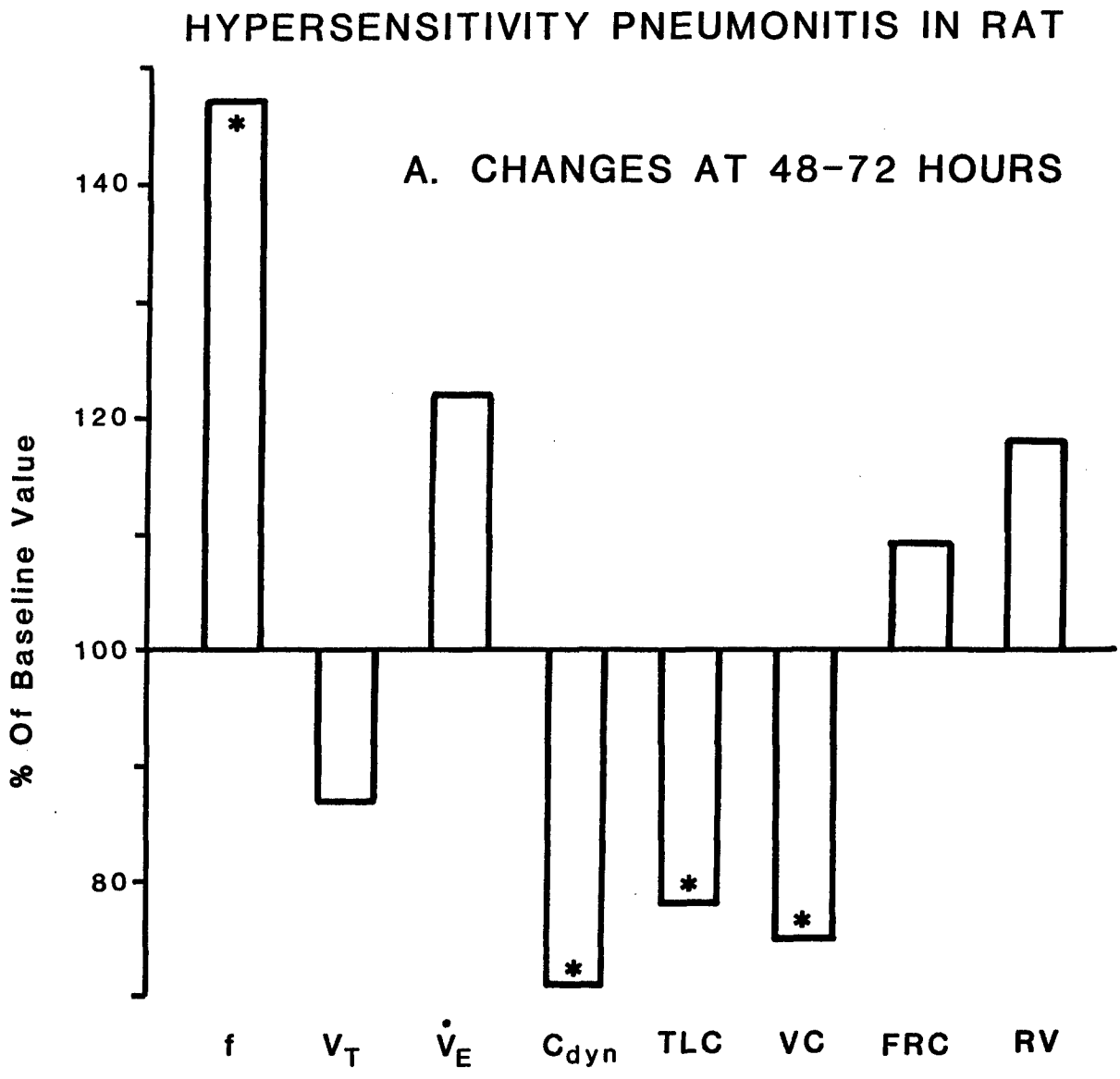

Figure 4A. Hypersensitivity pneumonitis in rat.

The changes in respiratory function of sensitized male, F-344 rats at 2-3 days after challenge with intratracheally-instilled ovalbumin are shown as percentages of pre-challenge values. Asterisks indicate changes significant at $P < 0.05$ as determined by Student's t test. A: Alterations in breathing pattern, dynamic lung compliance and lung volumes.

Values for C_{dyn}, TLC, and VC were reduced. Marked alterations in forced expiratory variables were present (Figure 4-B), including reductions in FVC, $FEV_{0.2}$, PEFR, FEF_{50} and FEF_{25} and a normal $FEV_{0.2}/FVC$. All of these alterations were typical of those observed in man with hypersensitivity pneumonitis.

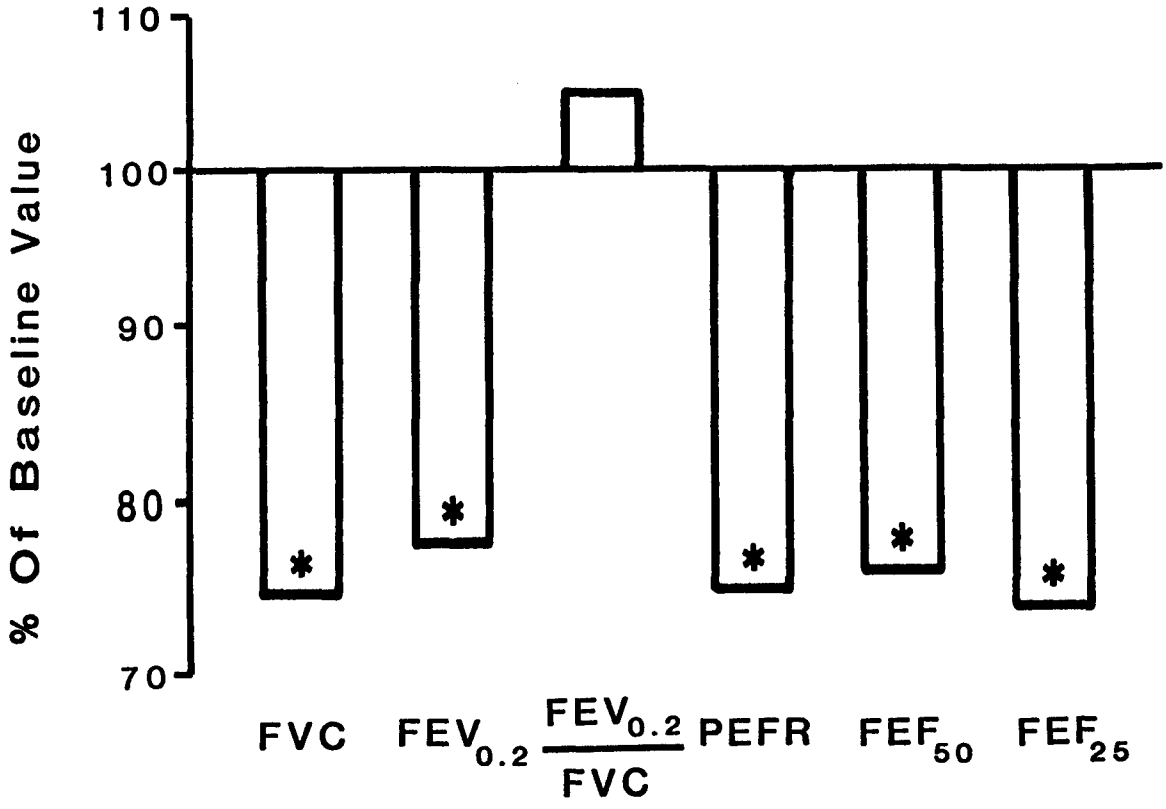

Figure 4B. Forced expiration at 48-72 hours.

The changes in respiratory function of sensitized male, F-344 rats at 2-3 days after challenge with intratracheally-instilled ovalbumin are shown as percentages of pre-challenge values. Asterisks indicate changes significant at $P < 0.05$ as determined by Student's t test. B: Alterations in forced expiratory parameters.

There are species and strain differences among animals in their responses to inhaled or instilled antigens after sensitization. There are variations among inbred rat strains in their ability to produce reaginic antibodies (Murphey et al., 1974). Strains which are good producers of reaginic antibodies would develop immediate (Type I) responses to antigen, while strains which are poor producers (such as the Fischer 344 rats at this Institute) would have delayed (Type III) responses. Sensitization of guinea pigs (strain

not specified) to ovalbumin and subsequent aerosol challenge was shown by Holroyde et al. (1980) to produce a Type I response with immediate functional changes which were reversed by administration of aminophylline. The relationships between strains, species, and types of immunologic responses in animals are not well understood; however, present data indicate that the resulting functional responses in animals are similar to those of man when the type of immunologic reaction is similar.

Acute, high level NO_2 inhalation

Humans have been accidentally exposed by inhalation in occupational settings to levels of NO_2 ranging from tens to hundreds of ppm. Although no controlled studies have been performed, the results of such exposure form a typical pattern (Jones, 1980). The severe sequelae usually occur hours to days after the inhalation episode, and are manifested clinically by dyspnea, coughing up mucoid sputum, and leukocytosis. Morphologic changes consist primarily of epithelial damage, edema, and hemorrhage. The most severe functional abnormality is a gas exchange impairment.

Jones et al. (1973) reported four cases of known or suspected occupational exposures to NO_2. Subject No. 2 in that report was a 35-year-old shipyard worker who developed symptoms after using an oxyacetylene torch in a poorly ventilated fuel tank. Although the exposure was not limited to NO_2, the clinical and functional alterations were typical. Initial symptoms were dyspnea and coughing of copious yellow sputum. On examination 2 hours after exposure, his respiratory rate was approximately twice normal (Figure 5-A), and radiographic changes were consistent with pulmonary edema. Treatment was initiated and the subject's condition improved until, on the fifth day, radiographs suggested that the clearing of edema was complete. The subject's gas exchange impairment was evaluated by measuring DLCO, and values on days 3, 6, and 13 after exposure were 56, 80, and 103%, respectively, of the predicted normal value (Figure 5-B).

Johnson et al. (1982) exposed dogs for 6 hours to 69 ppm NO_2 and evaluated clinical signs, respiratory function, and lung pathology serially for 14 days after exposure. Soon after exposure, the dogs developed a productive cough with yellow sputum and became dyspneic. The mean f was elevated to 239% of baseline at 2 hours after exposure (Figure 5-A), remained elevated through 2 days, and then returned to normal by day 7. The dogs had pulmonary edema with foam in the airways soon after exposure, and these changes lessened during the next few days. The edema caused a marked gas exchange impairment as illustrated by mean values for DLCO in Figure 5-B. The DLCO was reduced to 35% of baseline at 2 hours and returned to normal by 14 days after exposure.

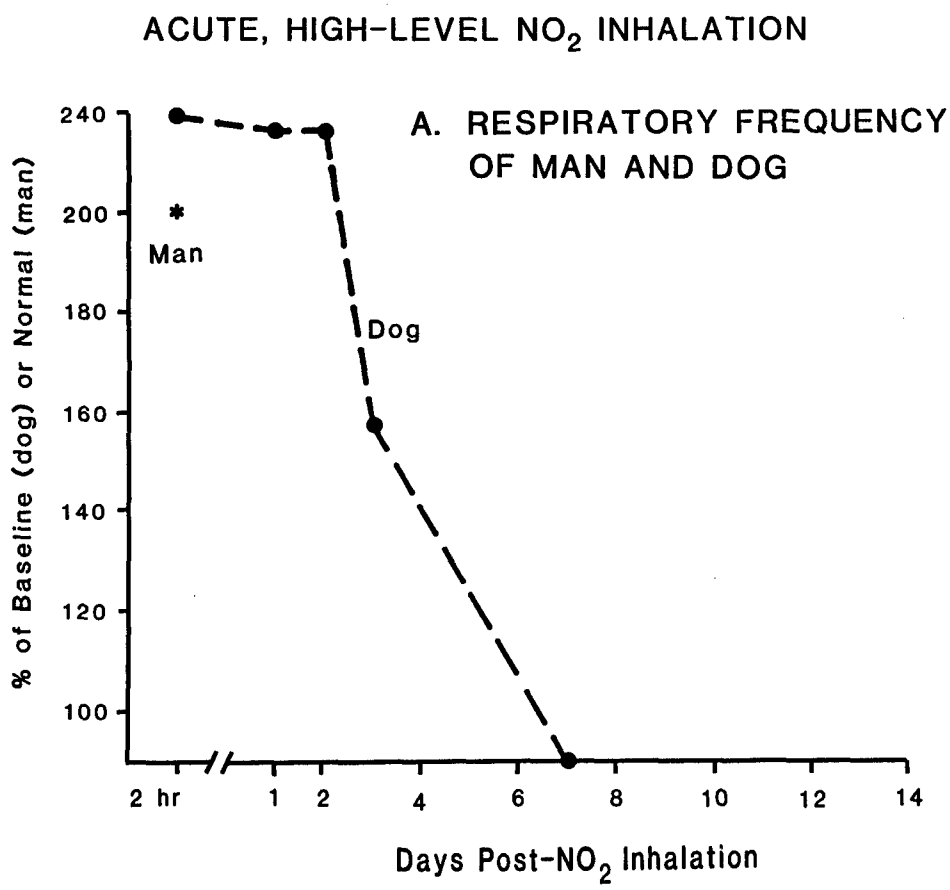

Figure 5A. Acute, high-level NO_2 inhalation.

Changes in the f (A) of a man after inhalation of welding fumes (Jones et al., 1973) and of dogs after inhalation of 69 ppm NO_2 for 6 hours (Johnson et al., 1982) are shown. Values are expressed as percentages of pre-exposure baseline (dog) or normal (man) values.

The similarities between the two species in their clinical, functional, and morphologic responses are striking. Although the changes shown in Figure 5 are even quantitatively similar, quantitative comparisons cannot be made because of the uncertain human exposure. The nature and time course of the clinical and functional changes of the human subject in this comparison were similar to those in other reports with known NO_2 exposures but less well-documented functional changes.

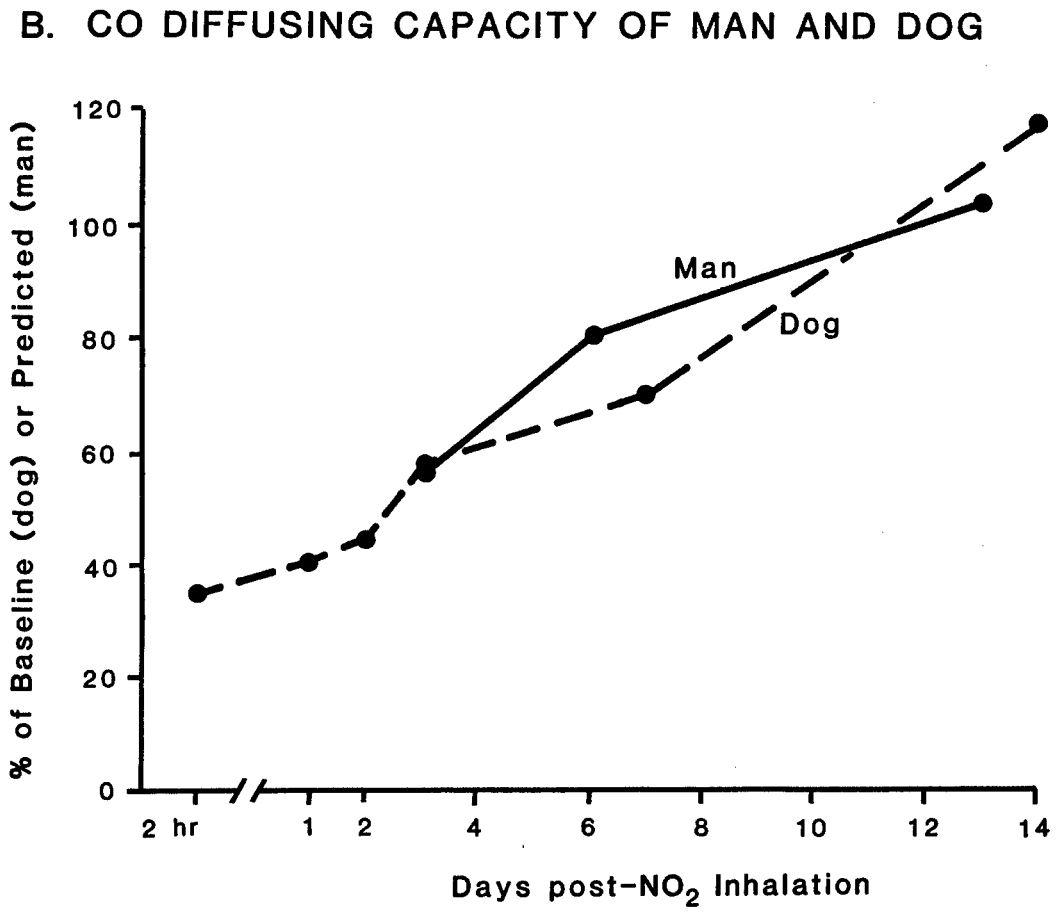

Figure 5B. CO diffusing capacity of man and dog.

Changes in the DLCO (B) of a man after inhalation of welding fumes (Jones et al., 1973) and of dogs after inhalation of 69 ppm NO_2 for 6 hours (Johnson et al., 1982) are shown. Values are expressed as percentages of pre-exposure baseline (dog) or normal (man) values.

Oxygen toxicity

Humans exposed by inhalation to 100% O_2 for periods greater than a few hours develop respiratory distress and functional alterations somewhat similar to those described for NO_2. Because of the concern for patients receiving O_2 therapy this disease has been well studied in both man and animals (Clark and Lambertsen, 1971). The structural changes are initially endothelial damage,

inflammation, and edema, followed by consolidation and atelectasis. Functional changes consist primarily of reductions in C_{dyn}, TLC, VC, and DLCO and increases in FRC and RV.

Caldwell et al. (1966) exposed 4 human volunteers to 98% O_2 at 1 atmosphere for 30-74 hours and evaluated respiratory function. Subject No. 2, a 28-year-old male, was exposed for 48 hours, and after exposure exhibited the functional changes from pre-exposure values shown in Figure 6. There were slight changes in TLC and VC, but DLCO and C_{dyn} (labeled C_L in Figure 6) were markedly reduced.

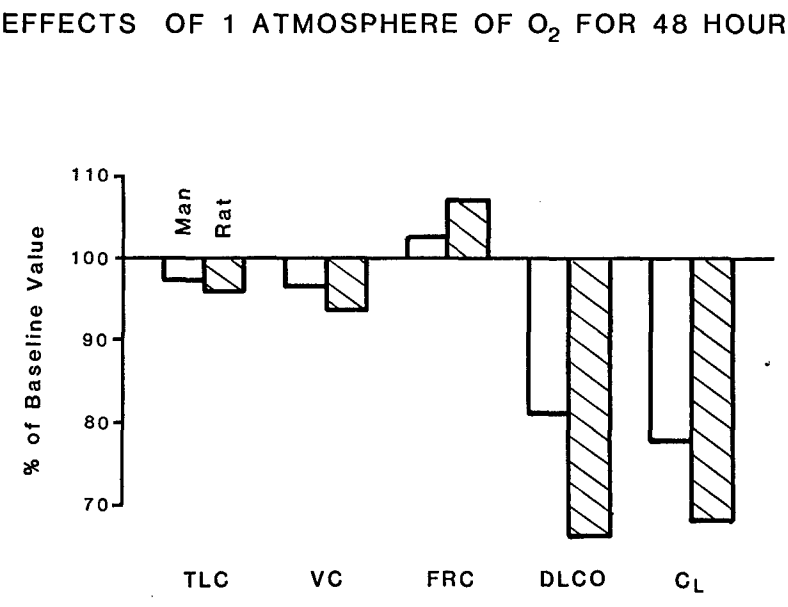

Figure 6. Effects of 1 atmosphere of O_2 for 48 hours.

Changes in respiratory function of a man (Caldwell et al., 1966) or rats (Harkema et al., 1982) after breathing one atmosphere of O_2 for 48 hours. Values for both species are expressed as percentages of pre-exposure baseline values.

Harkema et al. (1982) exposed 10, 4-month-old female Fischer 344 rats to 100% O_2 at 1 atmosphere for 48 hours and evaluated histopathology and respiratory function. The mean group changes from baseline values shown in Figure 6 are strikingly similar, both qualitatively and quantitatively, to those of the human subject. Since only one human subject is represented, and his changes lie within the range of individual rat changes, little significance can be drawn from the slightly greater magnitudes of changes in the rats.

CHRONIC RESPONSES

Radiation pneumonitis-fibrosis

Humans exposed to sufficiently large doses of thoracic irradiation, such as during the treatment of thoracic tumors, develop a restrictive type of lung disease that has been well studied in both man and animals (Rubin and Casarett, 1968). An initial inflammatory response is followed by a progressive interstitial fibrosis; the severity is both dose and dose rate dependent. The functional changes reflect the stiffer lung tissue, with reduced C_{dyn} and C_L, smaller lung volumes, and a breathing pattern altered toward a higher f and reduced V_T. This breathing pattern results in a greater proportional ventilation of V_D; therefore, the $\dot{V}_E$ is increased to maintain alveolar ventilation. Gas exchange is progressively impaired by the fibrosis, leading to a reduction of DLCO (typically the first functional parameter to be changed), an increase in $P_{A-a}O_2$ and, if sufficiently severe, to hypoxemia.

Emirgil and Heinemann (1961) studied the respiratory function of human subjects receiving unilateral thoracic irradiation therapy for malignant tumors. Subject No. 1 was a 31-year old female who received a total tumor dose of 5,000 roentgens of X-irradiation over a period of 39 days for treatment of a mammary carcinoma. Respiratory function was measured before treatment and later as she developed pneumonitis and fibrosis. At 233 days after treatment, the functional changes shown in Figure 7 were reported. The breathing pattern (f, V_T, and $\dot{V}_E$) was characteristically altered, the V_D/V_T ratio was increased, and a gas exchange impairment was demonstrated by a reduced DLCO and increased $P_{A-a}O_2$.

Mauderly et al. (1973) exposed dogs by inhalation to ^{90}Yttrium in fused aluminosilicate particles and measured respiratory function serially before and after the single, short-term exposure. A portion of the inhaled particles was retained in the lung and the dogs thus received whole-lung beta-gamma irradiation which, due to the half-life of ^{90}Yttrium, was delivered within 30 days of exposure. Dog 435B, a 14 month old male Beagle, received a total cumulative dose to the lungs of 9,900 rads, developed progressive pneumonitis and fibrosis, and died from respiratory failure at 131 days post-exposure. At 8 weeks after exposure (approximately 4 weeks after completion of dose delivery), the functional changes shown in Figure 7 were measured. These changes were both qualitatively and quantitatively similar to those of the human subject.

A further comparison was made between the post-irradiation changes in C_{dyn} of dog 435B and those of subject No. 3 in the report of Emirgil and Heinemann (1961). That subject was a 48-year-old female who received a tumor dose of 5,000 roentgens of x-irradiation for a mammary carcinoma. The authors did not report C_{dyn} values;

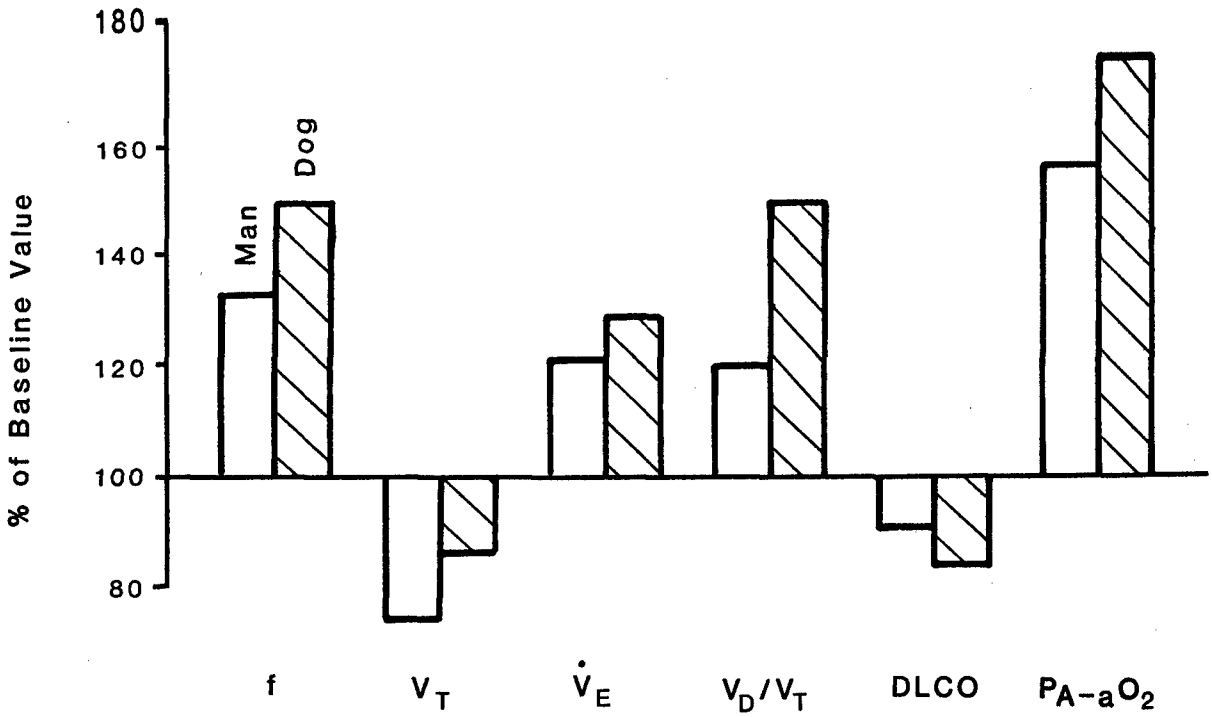

Figure 7. Radiation pneumonitis-fibrosis in man and dog.

Changes in respiratory function of a woman at 233 days after unilateral thoracic x-irradiation therapy (Emirgil and Hienemann, 1961) and of a male Beagle at 4 weeks after whole-lung irradiation from inhaled particles containing the beta-gamma emitter, ^{90}Yttrium (Mauderly et al., 1973). Values are expressed as percentages of baseline, pre-irradiation values.

however, they presented a figure showing tidal pressure-volume loops from which C_{dyn} was calculated for this comparison. The progressive reductions of C_{dyn} of the dog and human subject are compared in Figure 8. The C_{dyn} of both subjects declined in a parallel manner during the first 80-100 days following irradiation, presumably reflecting progressive fibrosis. The dog's C_{dyn} then declined more rapidly until death, while that of the human continued to decline, but less rapidly. These comparisons demonstrate that dogs and man have qualitatively similar respiratory function alterations in radiation-induced lung disease. The similarity of the responses is further supported by the reporting by Sweany et al. (1959) of post-irradiation reductions of TLC, VC, and RV of dogs, measurements not performed in the study by Mauderly et al. (1973). Similar volume changes were measured in the subjects of Emirgil and Heinemann (1961). In addition, DLCO has been reported to be the first

functional parameter to change in humans with radiation pneumonitis (Brady et al., 1965) and in dogs with radiation pneumonitis from inhaled ^{90}Yttrium (Mauderly et al., 1973) or ^{144}Cerium (Mauderly et al., 1980). Qualitative comparisons are not possible because of the differences in irradiation exposure of dogs and man in these studies.

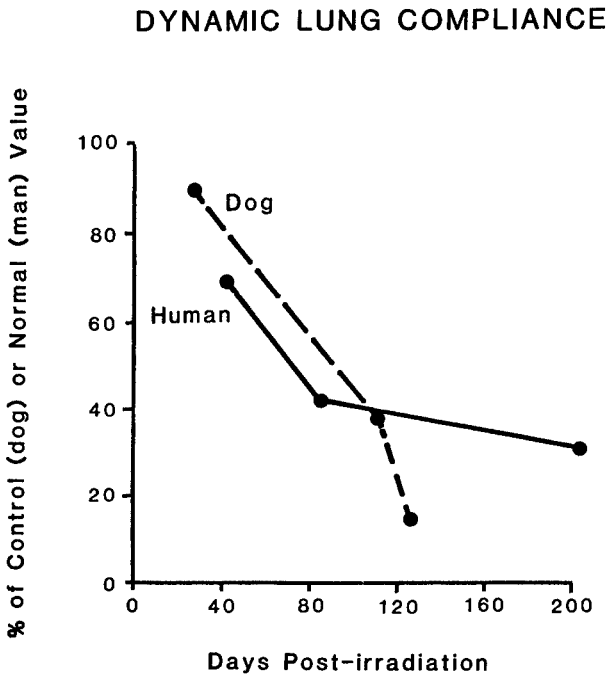

Figure 8. Dynamic lung compliance.

Changes in dynamic lung compliance of a woman after unilateral thoracic x-irradiation therapy (Emirgil and Hienemann, 1961) and of a male Beagle after whole-lung irradiation from inhaled particles containing the beta-gamma emitter, ^{90}Yttrium (Mauderly et al., 1973). Values are expressed as percentages of normal human and non-irradiated control dog values.

Pulmonary emphysema

Pulmonary emphysema is an abnormal enlargement of air spaces distal to terminal bronchioles with accompanying destructive changes of alveolar walls. Bates et al. (1971) give a thorough presentation of the anatomic, clinical, and respiratory function characteristics of the disease in man. The disease is most commonly accompanied by chronic bronchitis, as in smokers; thus, information from subjects with only emphysematous alterations is scanty. The primary

structural changes are destruction of alveolar walls and dilation of the terminal ventilating units. There is a loss of surface area for gas exchange and a loss of parenchymal support of small airways. This feature causes the major functional changes, consisting of increased C_L, FRC and RV, and airflow obstruction during forced expiration. The gas exchange impairment is related to the severity of the disease and ranges from negligible to severe.

Emphysema is not common among most animals, particularly the common laboratory species. Much effort has been directed toward the development of laboratory animal models of human emphysema, and this field has been well reviewed by Karlinsky and Snider (1978). Most investigators have treated animals with inhaled or intratracheally-instilled proteolytic enzymes which, after a transient period of inflammation, results in emphysematous lesions. In rodents, this model most closely resembles the panlobular or panacinar type of emphysema found in human cases of antiprotease deficiencies.

The respiratory function of Fischer 344 rats has been measured before and after intratracheal instillation of elastase in several current and previously reported studies at this Institute (Likens and Mauderly, 1982; Damon et al., 1982; Harkema et al., 1982). Likens and Mauderly (1982) found that all measured lung volumes of rats instilled with 1.0 IU/gm body weight were larger than those of controls at 4 weeks post-instillation. The mean TLC of 15, 3 month old, male, Fischer 344 treated rats, measured as the lung volume at 30 cm H_2O transpulmonary pressure, was 17.9 ml vs. 14.9 ml for 15 controls ($P < 0.002$). The relative magnitudes of lung volume subdivisions of the treated and control rats are shown in Figure 9. The most striking differences were the relative increases of FRC and RV with corresponding decreases in IC and VC. The increase in TLC reflected an increased lung compliance (also demonstrated in all studies as an increased C_L), and the increase in RV and TLC probably reflected an increased volume of trapped gas.

All studies have demonstrated characteristic alterations of forced expiratory parameters with a reduced FEV_{02} and flow rates over the entire volume range. These changes are illustrated by the flow-volume curves in Figure 10. Forced expiratory flow, induced by an airway pressure of -40 cm H_2O after lung inflation to TLC, is plotted against exhaled lung volume (FVC). Both curves are from adult male rats of nearly identical ages and weights. One rat received 1.0 IU/gm of elastase 4 weeks before the measurement, and its curve reflects lower flow rates throughout the exhalation. The concave or "dished out" appearance of the descending limb of the curve is typical. In all studies to date, forced expiratory parameters have been the most sensitive to emphysematous lesions in rats.

LUNG VOLUMES OF NORMAL AND EMPHYSEMATOUS RATS

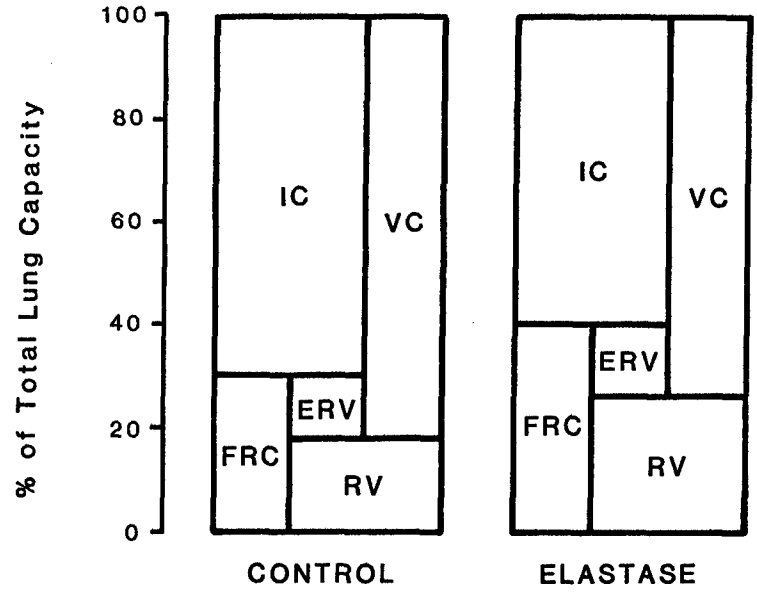

Figure 9. Lung volumes of normal and emphysematous rats.

The lung volume subdivisions of 15 male F-344 rats at 4 weeks after intratracheal instillation of 1.0 IU elastase/gm body weight and of 15 untreated control rats are expressed as mean percentages of total lung capacity (Likens and Mauderly, 1982).

The DLCO of rats with elastase-induced emphysema is variable, ranging from normal to significantly reduced, depending on the severity of lesions. Reductions of the mean DLCO of two groups of human subjects and two groups of rats with emphysema are compared in Figure 11. Six subjects studied by Thurlbeck et al. (1965) were described as having moderate emphysema with some degree of bronchitis and had a mean DLCO of 60% of the predicted value. Six male subjects ($\bar{x}$ age = 58 yrs) with a reduced $FEV_{0.75}$ and "thought to have emphysema" by Macklem et al. (1965), were found to have a mean DLCO of 61% of predicted. All of the human subjects had other functional changes consistent with the presence of emphysema. Nine rats from the study by Damon et al. (1982) were measured 3 weeks after receiving a mean dose of 1.0 (0.8-1.2) IU/gm of elastase and had a mean DLCO that was 75% of the control rat value. In a recent, unpublished study at this Institute, 11 rats instilled with 0.5 IU/gm elastase and measured 4 weeks later had a mean DLCO that was 95% of the corresponding control value. These results suggest a dose-response relationship in the treated rats.

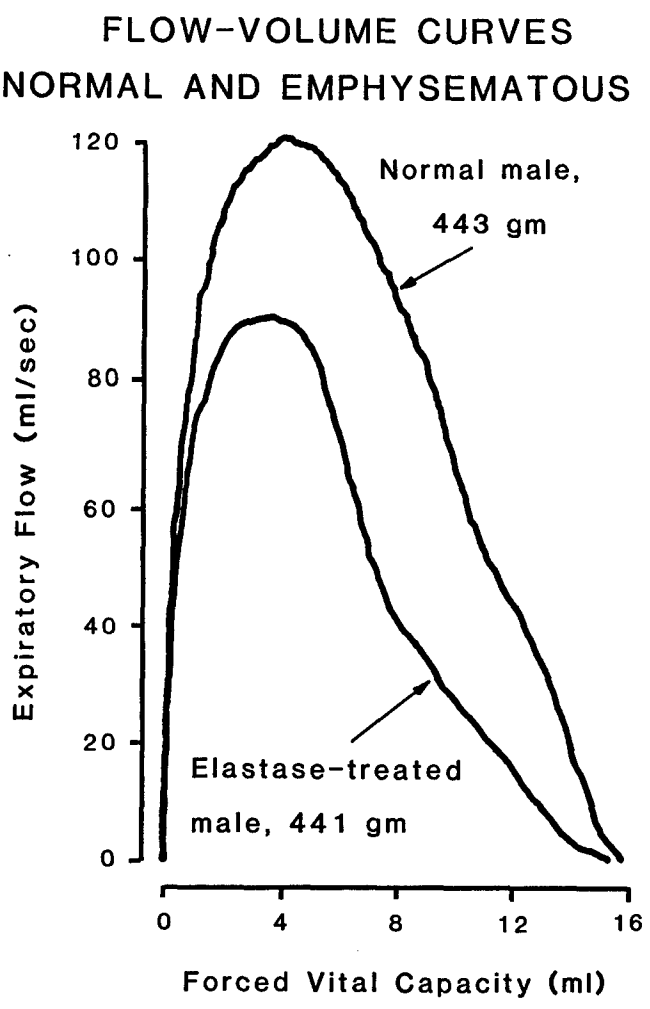

Figure 10. Flow-volume curves of normal and emphysematous rats.

Forced expiratory flow-volume curves of an adult male F-344 rat at 4 weeks after intratracheal instillation of 1.0 IU/gm body weight of elastase and of an untreated male rat are shown. The rats were hyperventilated to apnea, inflated slowly to total lung capacity and deflated rapidly at a negative airway pressure of 40 cm H_2O. Flow and volume were recorded at high speed on magnetic tape and later plotted at low speed on an x-y recorder.

These results indicate that rats and humans with pulmonary emphysema have qualitatively similar alterations of respiratory function, although differences exist due to testing methodology and the typical presence of other accompanying diseases in man. For example, the TLC was not increased in the human subjects of Thurlbeck et al. (1965), but Macklem et al. (1965) demonstrated an increased lung compliance at TLC in emphysematous man. If the human subjects had been anesthetized and inflated to a standardized transpulmonary pressure, and if their chest walls were as compliant as those of rats, their measured TLCs would undoubtedly have been

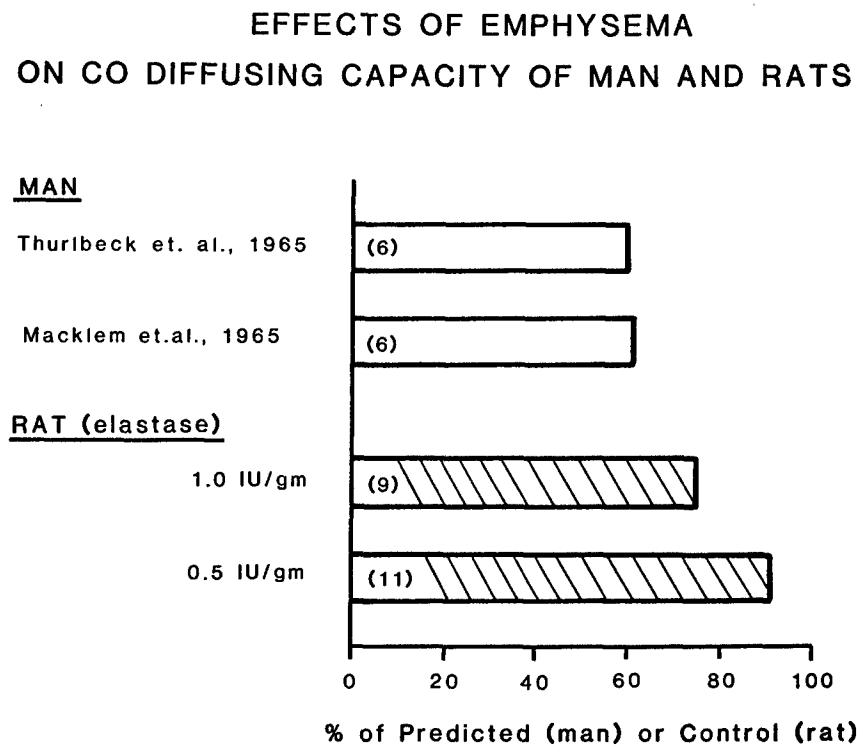

Figure 11. Effects of emphysema on CO diffusing capacity of man and rats.

The CO diffusing capacities of man and rats with pulmonary emphysema are compared. Numbers of subjects are shown in parentheses. Two groups of human subjects with other functional changes consistent with the presence of emphysema, but also having chronic bronchitis, had mean values that were 60% (Thurlbeck et al., 1965) and 61% (Macklem et al., 1965) of predicted. Two groups of young adult, F-344 rats intratracheally instilled 3-4 weeks previously with 1.0 (Damon et al., 1982) and 0.5 (previously unpublished) IU/gm body weight of elastase had mean values that were 75 and 91%, respectively, of control rat values.

increased as in the rats. This illustrates the need to keep testing methodology in mind when comparing results from different species, and indeed, from the same species measured by different methods. Interspecies differences are also likely to be affected by complicating diseases, as in the DLCO comparison.

SUMMARY

Respiratory function tests provide a strong link between experimental animals and man, because of the ability to perform physiologically similar measurements. Much is known about the relationships between measured functional abnormalities, subjective loss of function, clinical abnormalities, and occupational disability of man. Since respiratory function can be similarly quantitated in experimental animals, respiratory function data from animals comprise a pathway of extrapolation in the prediction of health effects of inhaled toxic materials in man.

Interspecies differences in pulmonary anatomy and in sensitivity to the induction of some types of functional abnormalities must be considered in relating data from animals to those from man. The ability exists, and is being continually extended, to interpret results from animals, but correct interpretation requires a good understanding of the specific methodologies employed and anatomical differences between species.

The data collected to date indicate that, if similar structural changes are induced in the lungs of animals and man, similar physiologic abnormalities will result. The manifestations of the underlying structural changes, as expressed in altered respiratory function values, may differ slightly among species; however, functional responses to lung injury appear to be similar in animals and man if they are correctly interpreted.

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PAPER NO. 13

COMPARATIVE BIOCHEMICAL RESPONSES TO INHALED TOXICANTS

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INTRODUCTION

Three years ago at this meeting I presented data on mucus glycoprotein biosynthesis obtained with rat tracheal slices suggesting that rats do not "adapt" to ozone exposure when assays not directly sensitive to inflammation/edema are used to define the lung's response to inhaled toxic substances. I suggested that the conventional biochemical measurements of enzyme levels in lung homogenates stressed by previous workers in inhalation toxicology might reflect cellular infiltration and serum transudation into the lung to a greater extent than such measurements served to reflect basic changes in lung cells per se. For the purpose of today's discussion of possible species differences in response(s) to inhaled toxicants I will again turn to the tracheal slice system for illustrative data, as well as to another system we have studied in greater detail, collagen synthesis by lung parenchymal minces. My basic conclusion will be that there may be quantitative differences between species in response to inhaled toxicants as measured by these parameters; furthermore, there may be potential analytical artifacts caused by species differences in the primary amino acid or sugar sequences of these structural proteins of the lung. Nonetheless, the basic biochemical response to ozone and to other inhaled toxins is much the same across species as diverse as laboratory rodents and primates. It is important to emphasize that this conclusion is based on evaluation of biosynthetic parameters that are basically lung-specific; i.e., potential confounding effects of inflammation/edema are minimized by the choice of methodology. Ozone-induced changes in lung-specific parameters persist over a chronic (months) time frame rather than becoming attenuated within a few days to a week, usually indicative of inflammation/edema-based response.

In the first set of experiments, we examined apparent secretion rates of mucus glycoproteins by tracheal slices cultured in the presence of radioactively labelled precursors (usually [^{3}H]glucosamine) from rats (Last et al., 1977) and bonnet monkeys (our unpublished data) exposed to ozone, as shown in Table 1. While there are quantitative and temporal differences in the response of rats and monkeys to ozone, the basic response is the same -- augmented

secretion rates in response to this irritant gas. We do not know whether this change reflects differences in biosynthetic ability of individual cells or is secondary to cellular hypertrophy and/or hyperplasia in submucosal glands and in the epithelial layer.

TABLE 1. RESPONSE OF RATS AND BONNET MONKEYS (MACACA RADIATA) TO OZONE EXPOSURE

Species	Duration of Exposure, Days	Glycoprotein Secretion Rate, % of Control Value	
		0.5 ppm Ozone	0.8 ppm Ozone
Bonnet monkeys	7	135*	177*
Rat	14	-	113
Rat	30	-	120*

* Significantly greater than control value (Student's t-test), i.e., $P < 0.05$. Tracheal slices from rats exposed 8 hours per day or from monkeys exposed 23.5 hours per day to the indicated concentrations of ozone were incubated for 22 hours in medium 199 (GIBCO) plus salts, buffer (pH 7.8), and antibiotics under an atmosphere of $O_2:CO_2:N_2$ (40:5:55) at 100% relative humidity, with 2.5 μCi of D-[6-^{3}H]glucosamine (10 ci/mmol) as a glycoprotein precursor. Secreted glycoproteins were quantitated as total acid-precipitable radioactivity in the culture medium.

We can study ozone-induced changes in mucus glycoprotein secretion by tracheal slices in more depth by separation and purification of such glycoproteins into sub-classes. Secreted glycoproteins labelled with [^{3}H]glucosamine are solubilized by brief digestion with papain (Kaizu et al., 1979), then further purified by chromatography on a small column of diethylaminoethyl (DEAE)-cellulose, an ion exchange chromatography technique, as shown in Figure 1. Glycoprotein fractions are eluted with a linear salt gradient of lithium chloride (LiCl) in acetate buffer, pH 4.4. Neutral and acidic glycoproteins are functionally defined by whether they pass through or are bound to such a column, respectively, in 50 mM acetate buffer.

We have examined the ratio of acidic to neutral glycoproteins (as defined by this DEAE-cellulose chromatographic technique) secreted by tracheal explants from various species as a probe of potential qualitative change in this parameter induced by inhaled toxins. There is a wide range of ratios of acidic to neutral glycoproteins (from about 2.5-7.5) secreted by tracheal slices from different species (Kaizu et al., 1979). There are also changes in this ratio with duration of incubation of slices in vitro (Kaizu et al., 1979). However, under standardized conditions this assay can be used as a probe for effects of inhaled toxins on mucus glycoprotein secretion by airway epithelia. Ozone exposure of rats may be used as a paradigm of such an assay system. For example, tracheal slices

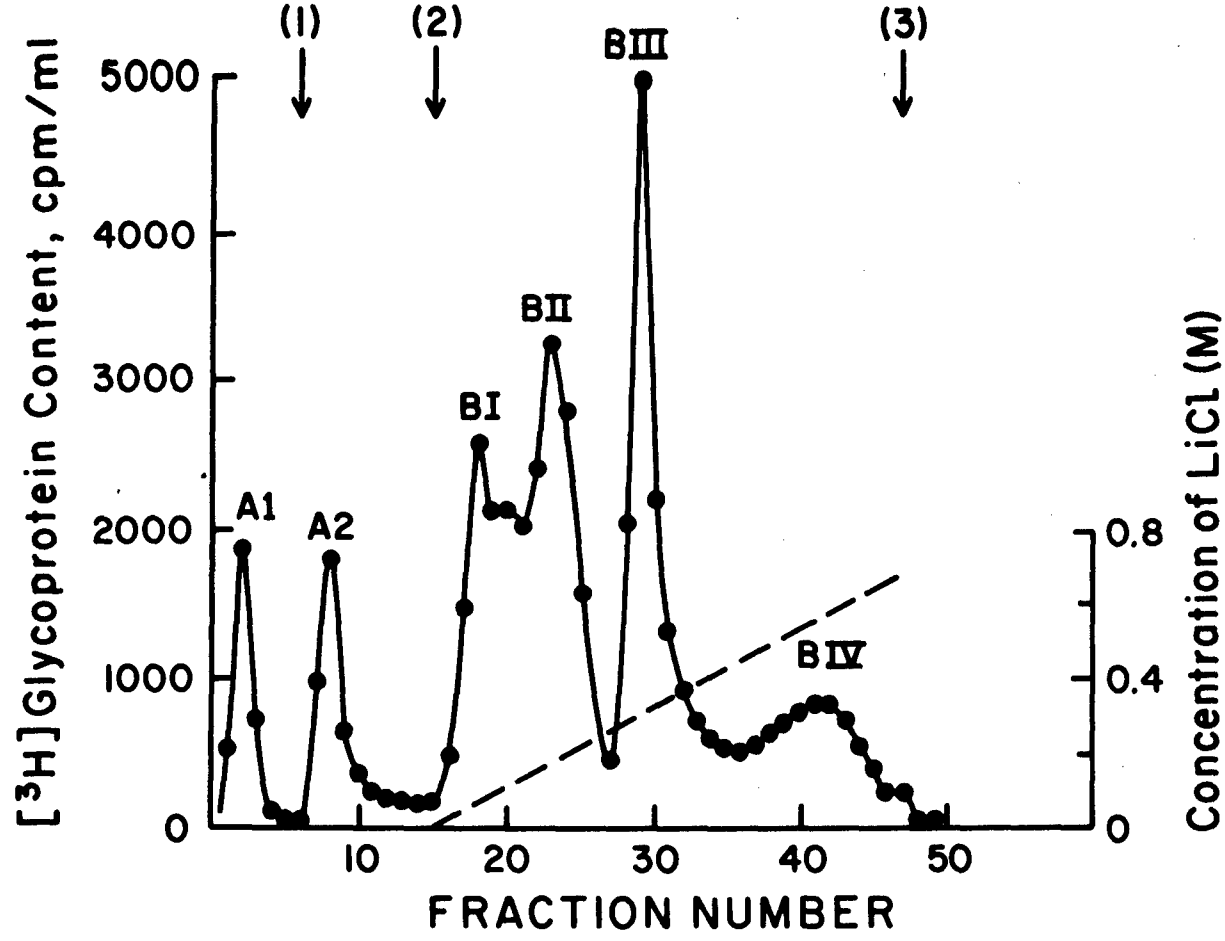

Figure 1. DEAE-cellulose chromatography of papain-treated mucus glycoproteins secreted into the culture medium by normal rat tracheal explants. Arrows indicate (1) 50 mM acetate buffer wash; (2) application of gradient; (3) 2 M LiCl wash. Fraction volume was about 2.2 ml; the radioactivity in a 1.0 ml aliquot was counted (solid line). The LiCl concentration is shown by the broken line, as determined by conductivity measurements (Radiometer, Copenhagen).

from control rats and from rats continuously exposed for 3 days to 0.4 ppm of ozone were incubated for 24 hours in vitro in the presence of [^{3}H]glucosamine. Secreted glycoproteins were solubilized and fractionated by ion-exchange chromatography on DEAE-cellulose (Kaizu et al., 1979; Last and Kaizu, 1980). The results of such an experiment (our unpublished data) are shown in Table 2. There is an apparent increase in the relative amounts of the glycoproteins that comprise peaks A and B IV at the expense of the B II glycoproteins in rats exposed to ozone. However, despite these

quantitative changes in glycoprotein classes secreted by tracheae of rats exposed to ozone, there are no apparent qualitative changes in the mixture of mucus glycoproteins being produced (i.e. the same peaks are observed upon chromatography).

TABLE 2. COMPARATIVE DEAE-CELLULOSE ANALYSES OF MUCUS GLYCOPROTEINS SECRETED BY TRACHEAL EXPLANTS FROM CONTROL AND OZONE-EXPOSED RATS

Peak	Mean % (±1SD) of Total Peaks	
	Control	Exposed
A1 + A2	10.9 ± 0.35	13.8 ± 0.80
B I	12.9 ± 1.2	14.8 ± 1.2
B II	37.9 ± 9.7	24.9 ± 6.5
B III	24.7 ± 7.0	22.0 ± 4.2
B IV	13.6 ± 3.1	24.5 ± 9.3
Ratio $\frac{\text{Acidic (B)}}{\text{Neutral (A)}}$	8.18 ± 0.31	6.28 ± 0.43

Acid-precipitable radioactivity in an aliquot of the culture medium after incubation of tracheal slices for 24 hours in the presence of 1.0 μCi of [^{3}H]glucosamine (10 Ci/mmol). The remaining medium was treated with papain to solubilize secreted glycoproteins (Kaizu et al., 1979; Last and Kaizu, 1980) and the resulting supernatant was fractionated in a column of DEAE-cellulose as shown in Figure 1.

A different type of species specificity in biochemical assays is exemplified by determination of quantitative and qualitative differences in collagen synthesis in vitro by lung minces from animals exposed in vivo to ozone. We have used this assay as a probe for acute responses to possible fibrogenic pneumotoxins; again, ozone exposure of rats may be used as a paradigm. Exposure of rats continuously for 1 week to high levels of ozone causes increases of 2- to 3- fold in apparent collagen synthesis rates by such lung minces (Last et al., 1979).

Interestingly, monkeys seem to be more sensitive to these same high levels of ozone by this assay; increases of 5- to 10-fold in apparent collagen synthesis rates by their lung minces are observed (Last et al., 1981). This apparent species difference is probably due to higher levels of ozone actually reaching the lung parenchyma of monkeys at a given concentration of ozone in air due to increased penetration of the nasopharyngeal defenses of monkeys because of their ability to inhale by mouth.

Quantitation of apparent collagen synthesis rates by lung minces is but one type of assay that may be performed for effects of inhaled toxic agents on parenchymal structure. Such an assay measures synthesis of total lung collagen, since all known collagens contain hydroxyproline. There are presently known to be about 12 distinct, genetically different collagen chains made by mammals. At least 5 types (composed of various combinations of collagen chains) of collagen are synthesized by lung minces and/or have been isolated from whole lungs. Types I and III together make up at least 90-95% of the total lung collagen and are the major structural proteins of the alveolar interstitium. Type II collagen is found in the cartilage surrounding large airways. Type IV collagen is a major component of basement membranes. Type V apparently occurs both in basement membranes and as an interstitial collagen. We can quantitate the relative ratios of various collagen types being synthesized by lung minces by use of fairly sophisticated biochemical techniques. In these assays the lung mince is digested with cyanogen bromide to break down collagens and generate soluble peptide fragments from the constituent insoluble collagen molecules present. By a combination of chromatographic and electrophoretic separation (Reiser and Last, 1980), we can isolate and quantitate index peptides from types I and III collagen, thereby allowing us to measure the relative ratios of these two classes of molecules being made by normal lungs or by those from rats exposed to inhaled toxicants. We find that there are qualitative differences in the collagens synthesized by lungs from rats exposed to ozone; the normal ratio of lung collagen types I and III is altered from 2:1 to about 5:1 in rats exposed to ozone (Reiser and Last, 1981). In fact, essentially all of the increase in collagen synthesis rate by the lung minces from rats exposed to ozone can be attributed to increased synthesis of type I collagen. However, in the course of trying to extend these measurements to lungs of silicotic horses (Schwartz et al., 1980) and other large animals, we found that the cyanogen bromide peptide that we used as a marker for type III collagen content (α1(III)-CB-8) had different molecular weights, and therefore different mobility upon polyacrylamide gel electrophoresis (Resier and Last, 1980), depending on the species examined (Reiser and Last[1]). In this case, the primary sequence of collagen varies in different species. The location of the various methionine residues in the collagen chains determines the exact peptide pattern produced by digestion of collagen with cyanogen bromide.

Several other fundamental properties of the mammalian respiratory tract are of potential importance when one considers possible species differences in responses to inhaled toxic substances. Nasal turbinates, airway branching, and the lung lining layer combine to trap particles (in a manner dependent on their size) and gases (in a manner dependent upon their aqueous solubility); these structural considerations may vary from species to species. For example, the

[1] Connective Tissue Research, in press.

rodent nose is a far more complex structure than that of the human or non-human primate, and thereby probably a better trap for particles. Rats also tend to breathe only through their noses (and indeed may be obliged to do so), whereas humans can (and do) breathe through their mouths, thereby bypassing their naso-pharyngeal defenses. Humans also have a better developed cough reflex than rats; hence, humans have a mechanism that may be largely unavailable to rats and mice for expulsion of particles from large conducting airways. Lobar anatomy of the rodent lung and different airway branching angles (as will be discussed by Dr. Tyler in this session) may result in different patterns of aerosol and particulate deposition in lungs of humans and rodents, with subsequent differences in observed effects. Finally, biochemical and structural changes in the lungs as a result of the lungs' history (exposure to cigarette smoke, bacterial pathogens, and/or environmental pollutants) may be a universal phenomenon for humans and for animals in nature, whereas lungs of the usual laboratory rodents have, in general, been protected from such prior insults. Thus, responses of the lung to inhaled toxicants may be modified by such prior insults or the lack thereof.

SUMMARY

In summary, two model systems are described for the study of effects of airborne toxicants on structural proteins of the lung: mucus glycoprotein synthesis by tissue slices from large airways and collagen synthesis by tissue minces prepared from lung parenchyma. The basic biochemical responses of mammalian lungs to inhaled substances seem to be universal; differences observed in such responses tend to be quantitative rather than qualitative. While potential species differences in the biochemical responses of lungs to inhaled toxic substances must always be considered, such species differences will usually be in the extent of response or reside in the detailed structure of the molecules involved, not in the response itself. Usually biochemical differences observed in response to toxic agents reflect underlying differences in the cellular content of the inflamed, acutely damaged lung, rather than necessarily reflecting changes in resident lung cells per se.

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PAPER NO. 14

GENERAL CONSIDERATIONS FOR DEVELOPING PULMONARY EXTRAPOLATION MODELS

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INTRODUCTION

The Clean Air Act is the basic U. S. Federal law for controlling air pollution in the United States. Determining the adequacy of existing National Ambient Air Quality Standards (NAAQS) or establishing new standards requires that the scientific information base be evaluated to assess pollutant effects on public health. Improvements in this process can be accomplished not only through new health effects research, but also through improved use of currently available data. To this end, this conference session on comparative responses to inhaled toxicants provides an opportunity to review information on species differences in respiratory toxicology which influence extrapolations to man for analyses of risk. The health information available on a given chemical usually arises from a variety of epidemiologic, human clinical, and animal toxicology studies.

It is immensely difficult for epidemiologic studies to quantitatively identify chemicals which cause disease because of the many confounding variables. While controlled human studies offer the ability to directly relate cause and effect in that exposure patterns can be accurately controlled and some confounding variables can be eliminated, they too have deficiencies. Because safety of the volunteer is of paramount importance, chronic exposures cannot be performed; only limited endpoints such as acute responses which can be measured with pulmonary function, clinical chemistry, or other non-invasive techniques, can be studied. This restricts the evaluation of pollutant effects to acute and typically reversible effects. While with human studies one can never investigate the full array of potential disease states induced by air pollutants, animal studies permit a complete evaluation of disease in that the

researcher has the choice of a wide range of concentrations, exposure regimens, chemical agents, biological measurements, and animal species.

Many physiologic mechanisms are common to animals and man so it can be hypothesized that if a pollutant causes a particular health effect in several animal species, it will be likely to cause similar effects in humans. However, quantitatively relating effective pollutant concentrations in animals to concentration-responses in man is extremely difficult. Efforts in this area can be considered to comprise "extrapolation modeling."

GENERAL CONSIDERATIONS FOR DEVELOPING PULMONARY EXTRAPOLATION MODELS

There are two major components to the pulmonary toxicologic evaluation of the effects of NAAQS pollutants in man and animals. One must be able to estimate the dose to the specific target region of the lung as a function of exposure concentration (dosimetry). Also, for a particular biologic endpoint, a damage function must be obtained which relates the effective delivered dose to the magnitude of the biologic response observed (sensitivity). For example, if the same dose is delivered to a target site in a given animal species and in a man, is the response equivalent? Information on both dosimetry and species sensitivity is vital for extrapolation of animal data to man.

DOSIMETRY

Extrapolation begins by estimating differences between man and experimental animals in the ratio of tissue dose to exposure concentration. To achieve this objective, greater emphasis must be placed upon developing mathematical models for pollutant regional deposition in the lung. These models should incorporate species anatomical and ventilatory differences and physicochemical properties of the pollutant as parameters and should be based upon the physical laws which govern transport and removal of the pollutant. The factors influencing the ability to estimate dose are different for gases as compared to particles.

Dosimetry for Particles

Physical Processes and Anatomical Considerations

For particles, the overriding factors influencing regional respiratory tract deposition are those based upon aerodynamic properties which, in turn, depend upon a variety of physical properties, including the size and shape of the particles and their physical densities. The aerodynamic diameter usually used is the

aerodynamic equivalent diameter (D_{ae}), defined as the diameter of a unit density sphere which has the same settling velocity (under gravity) as the particle in question of whatever shape and density (Hatch and Gross, 1964). Aerosols of two entirely different chemicals, such as lead oxide and cadmium oxide, will deposit similarly in the respiratory tract if their aerodynamic characteristics (i.e., mass median aerodynamic diameter and geometric standard deviation) are the same.

Attachment and irreversible removal of particles from the airstream results from contact of particles with moist airway walls. This contact can occur at any point in the breathing cycle or subsequently if a particle has been transferred to residual air (Davies, 1972; Engel et al., 1973). The primary physical processes leading to aerosol contact with airway walls are electrostatic attraction, interception, impaction, gravitational settling, and diffusion. The diffusivity and interception potential of a particle depend on its physical size, while the inertial properties of settling and impaction depend on its aerodynamic diameter. Depending on particle shape and physical density, these two measures of size can be quite different. The main mechanism of deposition is diffusion for particles less than 0.5 μm in aerodynamic diameter, while impaction and gravitational settling are important mechanisms for the deposition of particles greater than 0.5 μm D_{ae}. Electrostatic attraction and interception play minor roles in the respiratory tract deposition of most environmental aerosols, although the deposition of fibers is dependent mainly on interception.

When particles are inhaled, their aerodynamic properties, combined with various anatomic and breathing characteristics, determine their fractional deposition in various regions in the respiratory tract. The respiratory tract includes the passages of the nose, mouth, nasal pharynx, oral pharynx, epiglottis, larynx, trachea, bronchi, bronchioles, and small ducts and alveoli of the pulmonary acini. With respect to respiratory tract deposition and clearance of inhaled aerosols, three regions can be considered: (1) extrathoracic (ET), the airways extending from the nares down to the epiglottis and larynx at the entrance to the trachea (the mouth is included during mouth breathing); (2) tracheobronchial region (TB), the conducting airways of the lung from the trachea to the terminal bronchioles (i.e., that portion of the lungs having a ciliated epithelium); and (3) pulmonary region (P), the parenchymal airspaces of the lung, including the respiratory bronchioles, alveolar ducts, alveolar sacs, atria, and alveoli (i.e., the gas-exchange region).

Deposition Models

Models of the airways which simplify the complex array of branching and dimensions into workable mathematical functions are useful in comparing theoretical predictions of deposition with experimentally obtained deposition data. This can lead to more

refined models and an increased understanding of the processes which affect respiratory tract deposition. Findeisen (1935) developed a model of the airways of the human lung for estimating the deposition of inhaled particles. His model assumed branching symmetry within the lung, with each generation consisting of airways of identical size. Other models based on a symmetry assumption have been proposed (Davies, 1961; Landahl, 1950; Weibel, 1963). Asymmetric models more closely approximating the human lung have been developed by Weibel (1963), Horsfield et al. (1971) and Horsfield and Cumming (1968). Yeh and Schum (1980) have proposed a typical pathway lung model and have made particle deposition calculations for each lobe of the lungs. Although currently available particulate deposition models ignore the dynamic nature of the airways, future models should consider this aspect.

Historically, the most widely used models of regional deposition versus particle size were developed by the International Commission on Radiological Protection Task Group on Lung Dynamics (ICRP) under the chairmanship of P. E. Morrow (Morrow et al., 1966). These models were developed to determine radiation exposures from inhaled radioactive aerosols. Although the ICRP aerosol deposition and clearance models were not intended for broad application to environmental aerosols, they have been so applied by some scientists. Most model calculations treat the various mechanisms of deposition as independently occurring phenomena. However, such processes as Brownian diffusion and gravitational settling will interfere with each other when their effects are of comparable magnitude, such that the combined deposition can be less than the sum of the separate depositions (Goldberg et al., 1978). Taulbee and Yu (1975) have developed a theoretical deposition model which allows for the combined effects of the primary deposition mechanisms and features an imaginary expanding-tube model of the airway system (Weibel, 1963) based on cross-sectional areas and airway lengths. Also, a two component theory of aerosol deposition has been formulated (Yu, 1978).

At the time the ICRP Task Group models were developed, the available human data were primarily total deposition values for polydisperse and sometimes unstable aerosols (Brown et al., 1950; Dautrebande and Walkenhurst, 1966; Davies, 1964; Landahl and Black, 1947; Landahl and Herrmann, 1948; Morrow et al., 1958; Van Wijk and Patterson, 1940). Since then, in humans under different breathing conditions, total and regional respiratory tract deposition of monodisperse insoluble and stable aerosols of different sizes has been measured. Extensive studies have been conducted by Lippmann (1977), Heyder et al. (1975, 1980) Stahlhofen et al. (1980), Chan and Lippmann (1980), and Giacomelli-Maltoni et al. (1972). Additional useful data are reported by Palmes and Wang (1971), Shanty (1974), George and Breslin (1967), Altshuler et al. (1967, 1957), Hounam et al. (1971a, 1971b), Foord et al. (1976), Pavia et al. (1977), among others (Davies et al., 1972; Fry and Black, 1973; Heyder et al., 1975; Heyder and Davies, 1971; Heyder et al., 1973;

Hounam, 1971; Landahl et al., 1951; Landahl et al., 1952; Lever, 1974; Martens and Jacobi, 1973; Muir and Davies, 1967; Swift et al., 1977; Taulbee et al., 1978).

Nose breathing and mouth breathing provide contrasting deposition patterns for some respiratory tract regions. With nose breathing, nearly total respiratory tract deposition can be expected for particles larger than about 4 μm D_{ae}; mouth breathing bypasses much of the filtration capabilities of the extrathoracic region so that there is a shift upward to about 10 μm D_{ae} before there is complete deposition of inhaled particles (Environmental Protection Agency, 1982). However, given the three general regions into which the respiratory tract can be divided on the basis of anatomic structure, function, particle retention times, and clearance pathways, regional deposition data are more useful than total respiratory tract deposition information. Extrathoracic, tracheobronchial, and pulmonary deposition of particles in healthy human subjects who breathed through mouth pieces are shown in Figures 1, 2, and 3, respectively.

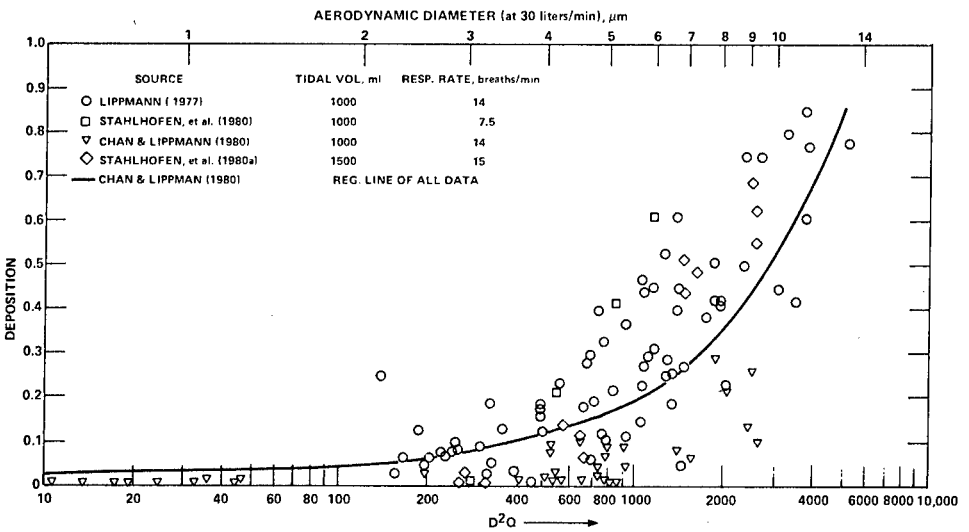

Figure 1. Deposition of monodisperse aerosols in the extrathoracic region for mouth breathing in man as a function of D^2Q, where Q is the average inspiratory flow rate in liters/min. The data are the individual observations as cited by the various investigators. The solid line is the overall regression derived by Chan and Lippmann (1980). The figure is from the Environmental Protection Agency, 1982.

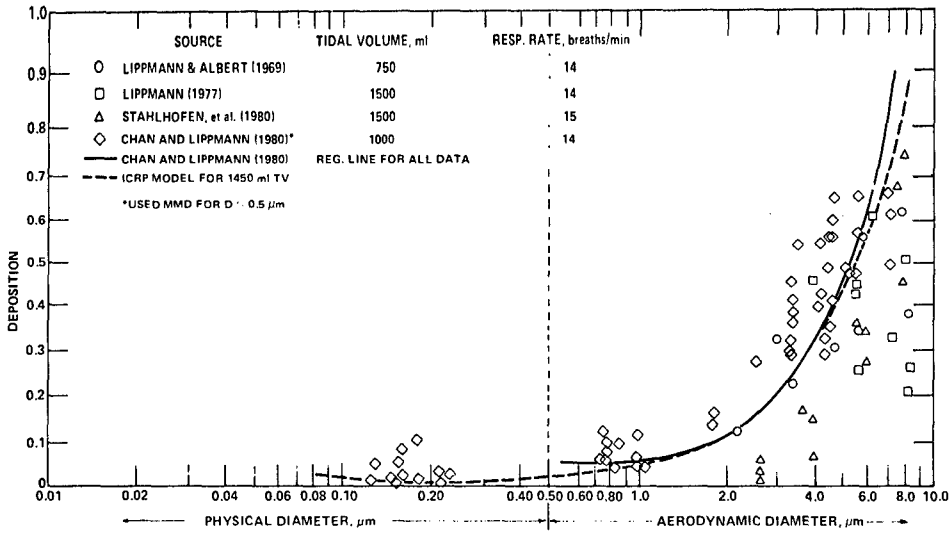

Figure 2. Deposition of monodisperse aerosols in the tracheobronchial region for mouth breathing in man in percent of the aerosols entering the trachea as a function of aerodynamic diameter, except below 0.5 µm where deposition is plotted versus physical diameter. The dashed line is the ICRP model (Morrow et al., 1966) for 1450 ml tidal volume. The solid line is the overall regression derived by Chan and Lippmann (Chan and Lippmann, 1980). The figure is from the Environmental Protection Agency, 1982.

Particles about 10 µm D_{ae} or larger are deposited in the extrathoracic region during nose breathing (Environmental Protection Agency, 1982). This compares to about 65 percent deposition of 10 µm D_{ae} particles under conditions of mouth breathing (Figure 1). On the other hand, extrathoracic deposition of particles smaller than about 1 µm D_{ae} is slight for both routes of breathing. The increased penetration of larger particles deeper into the respiratory tract with mouth breathing is reflected by experimental deposition data showing that tracheobronchial deposition of 8-10 µm D_{ae} particles is greater than 20 percent (Figure 2). Also, about 10 percent of particles as large as 15 µm D_{ae} are predicted to enter the tracheobronchial region during mouth breathing (Miller et al., 1979).

Inhaled particles with aerodynamic diameters less than about 4 µm have pulmonary deposition fractions between 20 and 70 percent. For mouth breathing, as compared to nose breathing, the peak of the pulmonary deposition curve shifts upward from about 2.5 µm D_{ae} to 3.5 µm D_{ae} (Figure 3). The peak is much less pronounced for nose

breathing (about 25 percent compared to about 50 percent for mouth breathing) with a nearly constant pulmonary deposition of about 20 percent for all sizes between 0.1 μm and 4 μm D_{ae}. Depending upon the tidal volume and breathing frequency, pulmonary deposition of particles 5 μm in aerodynamic diameter can vary from as little as 5 percent to as much as 50 percent. With mouth breathing, about 5-13 percent of particles 8-9 μm in aerodynamic diameter are deposited in the pulmonary region.

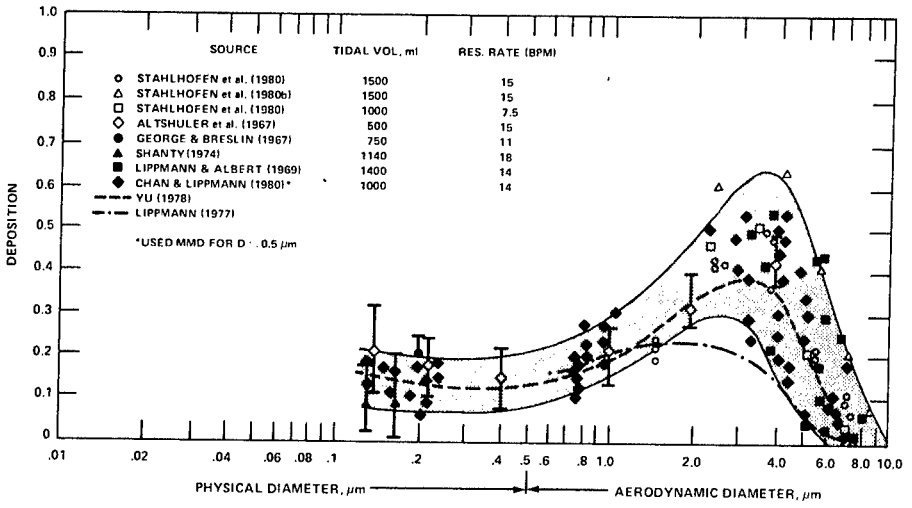

Figure 3. Deposition of monodisperse aerosols in the pulmonary region for mouth breathing in man as a function of aerodynamic diameter, except below 0.5 μm where deposition is plotted versus physical diameter. The eye-fit band envelops deposition data cited by the different investigators. The dashed line is the theoretical deposition model of Yu (1978) and the broken line is an estimate of pulmonary deposition for nose breathing derived by Lippmann (1977). The figure is from the Environmental Protection Agency, 1982.

Regional deposition of particles less than 3 μm D_{ae} in nose-breathing rats and hamsters is shown in Figure 4. Deposition of these size ranges has also been studied in dogs (Cuddihy et al., 1973; Phalen and Morrow, 1973). In these animal species, the relative distribution of particles less than 3 μm D_{ae} during nose breathing among the respiratory tract regions follows a pattern that is similar to regional deposition in man during nose breathing. Thus, in this instance, the use of rodents or dogs in toxicologic research for extrapolation to humans entails differences in regional deposition of insoluble particles less than 3 μm D_{ae} that can be

reconciled from available data. Before similar relationships can be determined for larger particles and other animal species, additional research needs to be conducted.

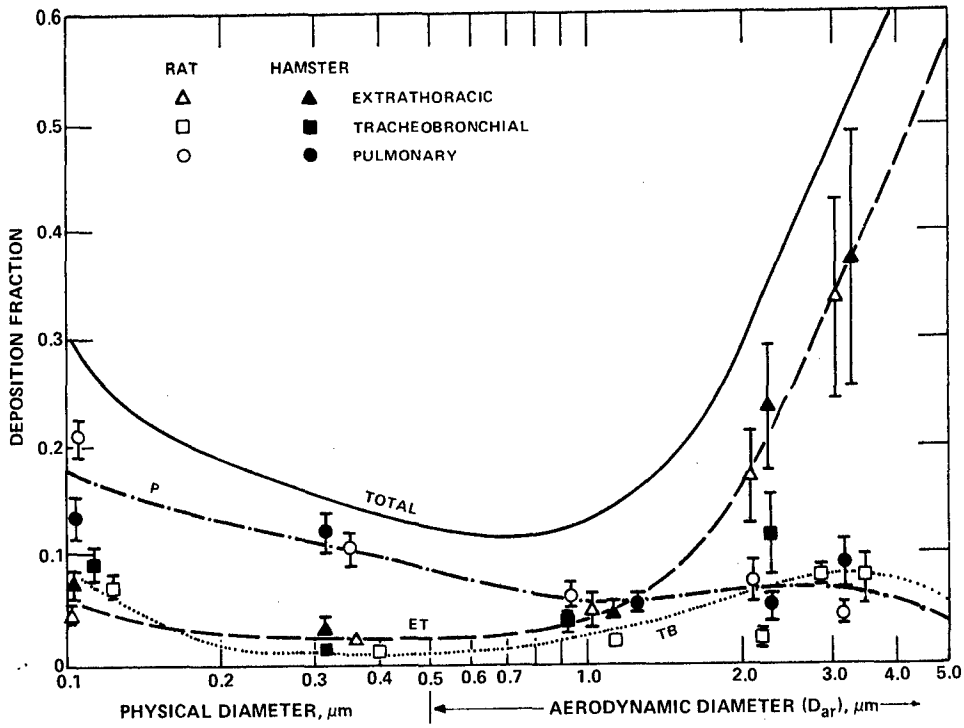

Figure 4. Deposition of inhaled monodisperse aerosols of fused aluminosilicate spheres in small rodents showing the deposition in the extrathoracic (ET) region, the tracheobronchial (TB) region, the pulmonary (P) region, and in the total respiratory tract based upon Raabe et al. (1977). The figure is from the Environmental Protection Agency, 1982.

Species Dosimetric Comparisons

The following illustrates how dosimetric comparisons can be made from an animal toxicologic study in which rats are exposed to an aerosol characterized by a given mass median aerodynamic diameter (MMAD) and geometric standard deviation (S_g). Using the regional deposition data given in Figures 1-4 and other deposition data cited earlier, one can integrate the product of the deposition and aerosol distribution curves to compute the mass of aerosol deposited in a given region of the lung. These estimates can be adjusted for ventilatory (Comroe et al., 1970; Crosfill and Widdicombe, 1961) and lung tissue surface area (Gil and Weibel, 1972; Kliment, 1973;

Weibel, 1963) differences to obtain comparisons of dose between rats and man, where dose is expressed as μg aerosol/cm^2 of lung-unit time.

Tracheobronchial and pulmonary region burden ratios (man/rat) for various MMADs are given in Figures 5 and 6, respectively. Effects on the burden ratio of variations in the standard deviation of the aerosol are also illustrated in the figures. If a man and a rat both breathed the same concentration of an aerosol, the tracheobronchial region of man would receive less of a burden than that of a rat for MMADs less than about 5 μm. Exactly how much less is a function of the particular MMAD, ranging from about 0.1 at 0.5 μm MMAD to 0.92 at 5 μm MMAD (Figure 5). For MMADs greater than 5 μm man receives more of a burden than does a rat, although caution should be exercised for larger MMADs since definitive deposition data for larger particles are lacking for the rat.

Pulmonary region body burden ratios (Figure 6) yield some interesting observations concerning the extrapolation of toxicologic results obtained in studies with rats to the likelihood of similar results being observed with human exposure. For aerosol exposures with MMADs below about 1 μm, the burden ratio (man/rat) is not sensitive to variations in the standard deviation of the aerosol. MMADs <2.5 μm yield lower burden ratios for man as compared to the rat. If sensitivity to a given dose were equivalent in man and rats, the toxicologic results from the rat study would be quantitatively directly extrapolatable to man. The nonavailability of the full range (0-15 μm D_{ae}) of particle size regional deposition data for man and several species of animals is the major factor which currently prevents the widespread formulation of such dosimetric relationships.

Clearance of deposited particles also influences dosimetry as related to extrapolation. The chemical solubility of the gas or the particle is a prime determinant of the rate of clearance. The more rapid the clearance, the less time available for adverse effects at the site of original deposition. Experiments related to the above dosimetric factors have been performed for other purposes, but this body of work needs to be reviewed and analyzed to extract data relevant to extrapolation models. For a general discussion on the factors influencing nasal, tracheobronchial, and alveolar clearance and on retention models and translocation of particles, the reader is referred to Raabe (1982).

Dosimetry for Gases

While aerodynamic properties provide a commonality for examining particulate deposition, gaseous deposition is more specific to the properties of the individual gas. The major factors affecting the uptake of gases in the respiratory tract are the morphology of the respiratory tract, the route of breathing, the depth and rate of

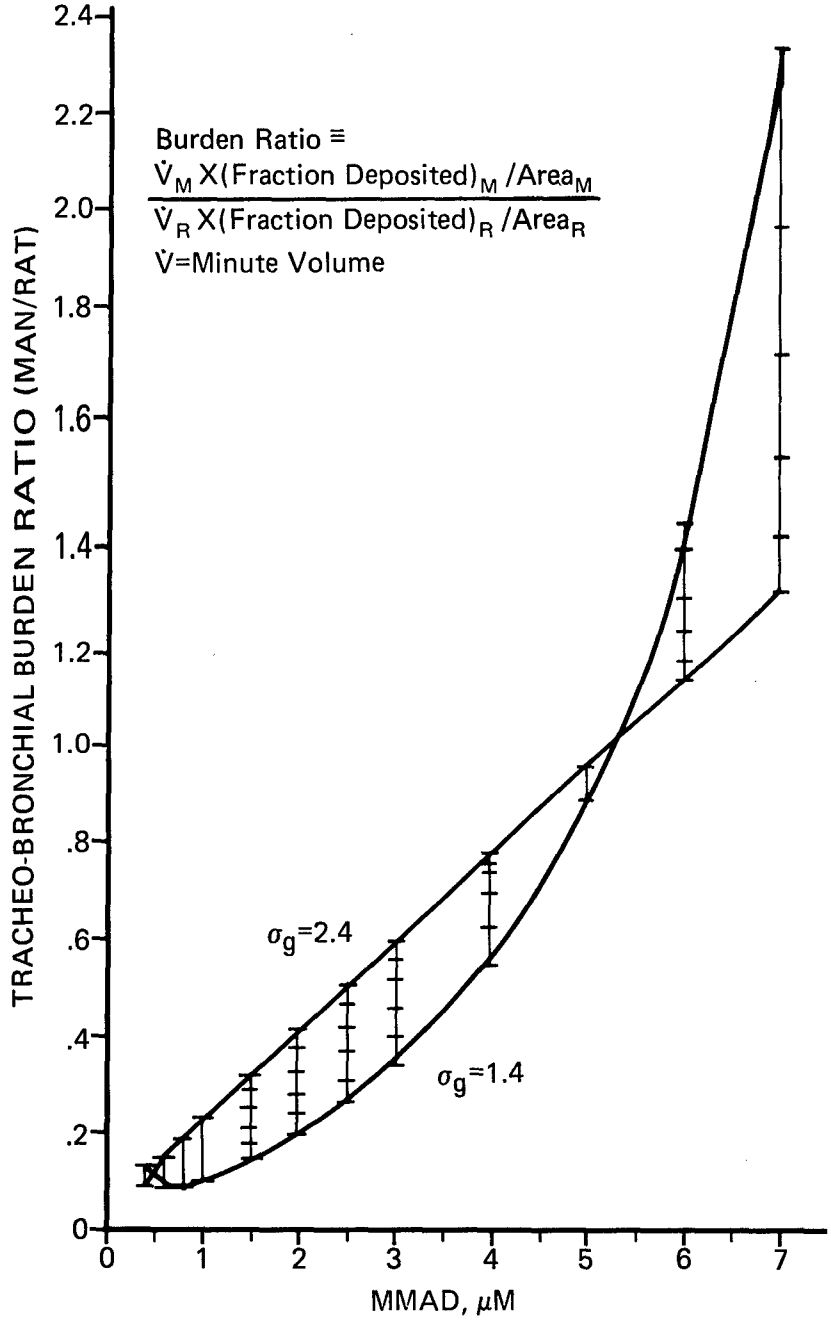

Figure 5. Tracheobronchial region burden ratio (man/rat) as a function of MMAD and S_g. The burden ratio is defined as

$$\frac{\dot{V}_M \text{ X } (\text{Fraction Deposited})_M \text{ / } \text{Area}_M}{\dot{V}_R \text{ X } (\text{Fraction Deposited})_R \text{ / } \text{Area}_R}$$

where $\dot{V}$ = minute ventilation, M = man, R = rat.

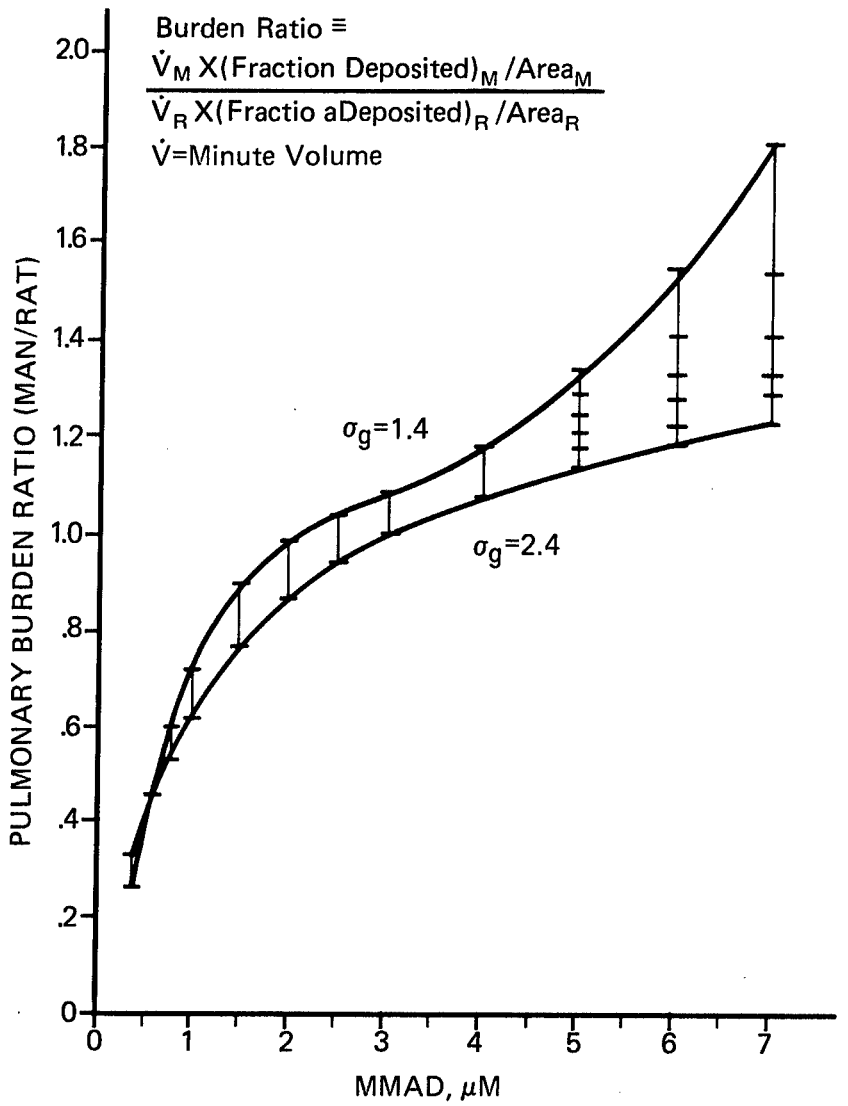

Figure 6. Pulmonary region burden ratio (man/rat) as a function of MMAD and S_g. The burden ratio is defined as

$$\frac{\dot{V}_M \text{ X (Fraction Deposited)}_M \text{ / Area}_M}{\dot{V}_R \text{ X (Fraction Deposited)}_R \text{ / Area}_R}$$

where $\dot{V}$ = minute ventilation, M = man, R = rat.

breathing, physicochemical properties of the gas, the physical processes which govern gas transport, and the physicochemical properties of the aqueous material on the surface of the airways or gas exchange units.

Physical Processes and Anatomic Considerations

The physical processes which govern gas transport involve convection, diffusion, and chemical reactions. The bulk movement of inspired gas in the respiratory tract is induced by a pressure gradient and is termed convection. Molecular diffusion due to local concentration gradients is superimposed on this bulk flow at all times, with the transport of the gas being accomplished by the coupling of these two mechanisms. Convection can be decomposed into the processes of advection and eddy dispersion (Butcher and Charlson, 1972). Advection is the horizontal movement of a mass of air that causes changes in temperature or in other physical properties, while eddy dispersion occurs when air is mixed by turbulence so that individual fluid elements transport the gas and generate the flux. Due to the morphology of the respiratory tract and respiratory airflow patterns, the relative contribution of the various processes to transport and deposition is a function of location and point in the breathing cycle.

In the tracheobronchial tree with its many branches, changes in caliber and irregular wall surfaces, it is difficult to establish exactly where flow is laminar, turbulent, or transitional. Viscous forces predominate in laminar flow and streamlines persist for great distances, while with turbulent flow there is rapid and random mixing. As the flow rate increases, unsteadiness develops and separation of the streamlines from the wall can occur leading to the formation of local eddies. This type of flow is termed transitional. The Reynolds number, the ratio of inertial to viscous forces, is useful in describing whether flow is laminar or turbulent. In smooth walled tubes, values between approximately 2000 and 4000 are ascribed to transitional flow with smaller Reynolds numbers reflecting laminar flow and larger ones turbulent flow. Fully developed laminar flow probably only occurs in the very small airways; flow is transitional in most of the tracheobronchial tree, while true turbulence may occur in the trachea, especially during exercise when flow velocities are high (West, 1977). The issue of turbulence is further complicated by the fact that for flow in a curved tube a Reynolds number greater than 7000 is apparently needed for turbulent flow (Taylor, 1929).

Deposition Models

In studying the nature of gas mixing in the tracheobronchial tree and its effects on gas transport, there have been numerous modeling efforts utilizing an approach in which all pathways from the mouth or trachea to the alveoli are combined into one effective pathway whose cross-sectional area is equal to the summed cross-sectional area of all bronchial tubes at a given distance from the mouth or trachea (Davidson and Fitz-Gerald, 1974; Paiva, 1973; Pedley, 1970; Scherer et al., 1972; Yu, 1975). In this formulation, the mechanical mixing imparted by tube bifurcations, turbulence, and

secondary flows and the mixing due to molecular diffusion are represented by the functional form of the effective axial diffusion coefficient (Scherer et al., 1975). Thus, this coefficient of diffusion incorporates the effect of axial convection. Since flow patterns at tube bifurcations are different for inspiration and expiration (Schroter and Sudlow, 1969), the mixing process and hence the effective diffusivities are different. To obtain diffusivities applicable to the tracheobronchial tree, Scherer et al. (1975) used airway lengths and diameters from Weibel (1963) and branching angles from Horsfield and Cumming (1967) to construct a five-generation symmetrical branched tube model and to experimentally determine effective axial diffusivity for laminar flow of a gas as a function of mean axial velocities up to 100 cm/s in the zeroth generation tube. The relationship was approximately linear and diffusivities for expiration were about one-third those for inspiration. The values obtained by Scherer et al. (1975) for steady flow can be applied to oscillating flow in the tracheobronchial tree provided the oscillating flow can be considered quasi-steady, i.e., steady at any instant of time. This condition should hold in the first ten generations whenever flow rates are approximately greater than 0.1 L/s (Jaffrin and Kesic, 1974).

By contrast to the expanding cross-sectional area geometry used for modeling axial diffusion, the appropriate geometry for radial diffusion is that of the airway lumen which has a decreasing radius proceeding distally from the trachea. Radial diffusion must be coupled with a scheme for incorporating any chemical reactions to account for the removal of the gaseous pollutant from the lumen of an airway. At this point the dosimetric modeling becomes specific to a given gaseous pollutant in that the determination of any chemical reaction that should be modeled is dependent upon the biochemical composition of the mucous and surfactant layer. Discussions of general considerations useful in modeling respiratory tract transport and absorption of gases are available (Chang and Farhi, 1973; Dubois and Rogers, 1968; National Research Council, 1976).

An approach to modeling gaseous deposition in the lung is illustrated by the work of Miller and coworkers (Miller et al., 1978) on the pulmonary uptake of O_3 and of NO_2 (Miller et al., 1982). Based upon the biochemical composition of mucus and surfactant, these investigators identified specific components of the mucous layer which undergo an irreversible instantaneous reaction with O_3. The airway lumen O_3 concentration determined the location in the mucous layer of the reaction plane between O_3 and the mucous reaction components. There is less certainty as to the appropriate reaction scheme for NO_2 and various components in mucus or in surfactant than there is for O_3. While it appears that NO_2 should react with many of the same constituents of the protective layers of the lungs as does O_3, it is possible that such reactions are not diffusion controlled, as is the case for O_3 (Menzel, 1976). Definitive studies are critically needed on the types and rates of reaction of NO_2 with various free amino acids, lipids, carbohydrates,

etc. Nevertheless, one can obtain an idea as to the effect of chemical reactions by using the instantaneous reaction scheme and varying the reactant production rate.

Results of simulations of this type are illustrated in Figure 7 for the NO_2 lost by the lumen (Panel A) and the tissue dose of NO_2 (Panel B) for each generation for a tracheal NO_2 concentration of 800 $\mu g/m^3$. K_o indicates a reference reaction production rate the same as that used for O_3. The factor in front of K_o refers to the fraction of K to which the reactant production rate has been reduced. Thus, the results are plotted of what may be considered extremes for the uptake of NO_2 - no chemical reactions ($0K_o$) and an upper limit, as represented by the O_3 rate ($1K_o$).

From Figure 7 it can be seen that the uncertainty of chemical reactions gives rise to a factor of five uncertainty in the uptake of NO_2 in the trachea (0th generation). The uncertainty decreases to insignificance by generation 14 or 15, and for the remaining generations (16-23) the uptake is not sensitive to the variation in the NO_2 reactant production rate.

Only a fraction of the NO_2 lost by the lumen may reach the tissue walls. This amount, the tissue dose, is plotted in Figure 7. A comparison of Figures 7A and 7B illustrates the protective nature of the mucous layer; with our values for the chemical reactions, no NO_2 reaches the tissue wall before the 12th generation (Figure 7B). For the reference curve ($1K_o$) over 14 times as much NO_2 is lost by the lumen (in the 14th generation) as penetrates to the tissue wall. This difference ceases at the 17th generation where, in our model, the protective layer has no reactants. From generation 17-23 the wall uptake is not only insensitive to the chemistry in the previous generations, but is the same as is lost by the lumen (Figure 7A).

Species Dosimetric Comparisons

Since the tissue dose curves presented in Figure 7B for $0.5K_o$ and $1K_o$ as a function of airway generation in the human lung are qualitatively similar, tissue dose curves comparisons for NO_2 in man compared to animals will be presented using a common value of $0.5K_o$.

For a tracheal NO_2 concentration of 800 $\mu g/m^3$, estimates for the dose delivered to the tissue in various generations of the human lung (Δ-Δ in Figure 7B) predict that NO_2 does not penetrate to the tissue prior to the 12th generation. This region represents one of the first branchings of the conducting airways near the terminal bronchioles. The dose curve then increases sharply, dips at the 16th generation, then reaches an absolute maximum at the first generation of respiratory bronchioles (generation 17). Proceeding distally there is a rapid fall in the dose of NO_2 delivered to the tissue, due in large part to the correspondingly rapid increase in

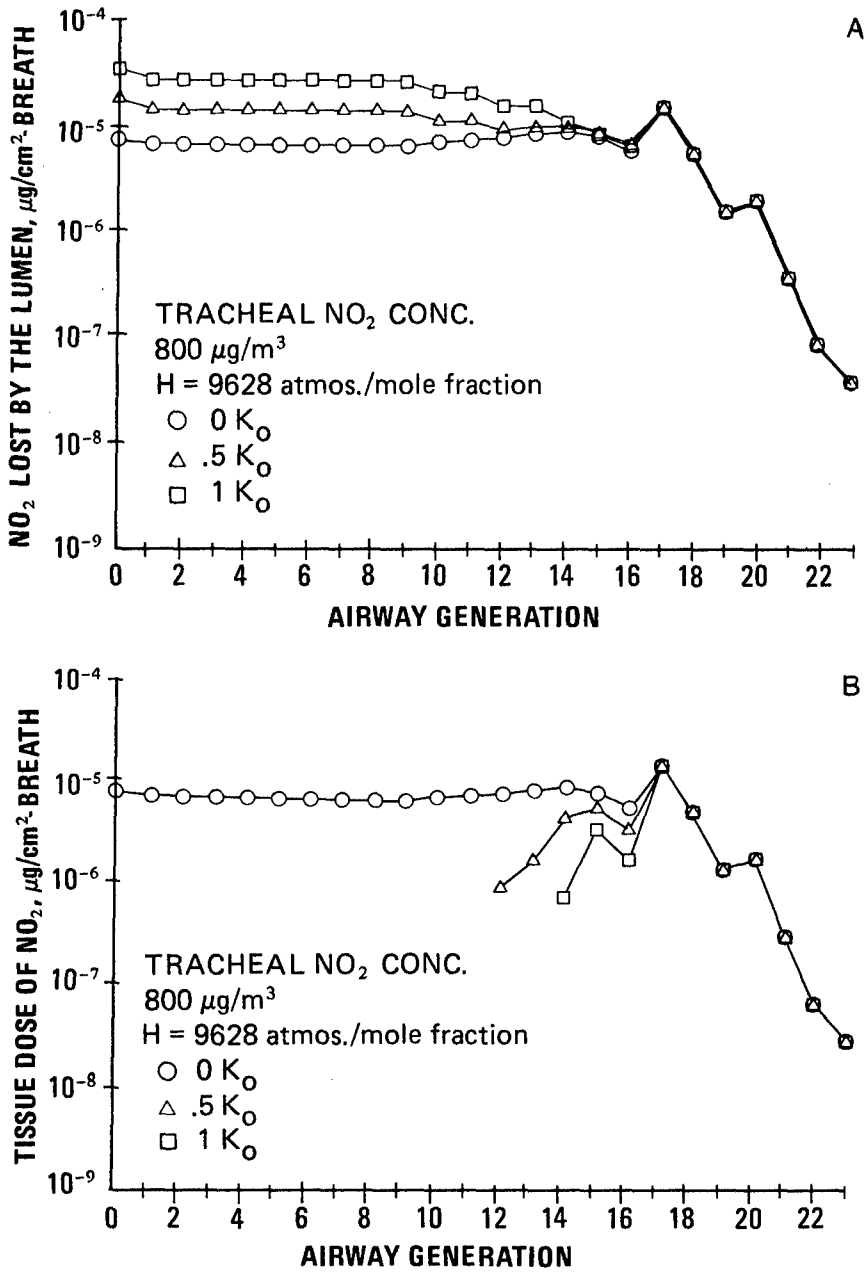

Figure 7. A. NO_2 lost by the airway lumen as a function of airway generation in the human lung.

B. Predicted NO_2 tissue dose curves in the lungs of man. Simulations used a tidal volume of 500 ml and a respiratory frequency of 15 breaths/min. K_o indicates a reference reaction production rate the same as that used for O_3.

lung surface area. Non-zero tissue doses in the conducting airways are approximately 3 to 15 times smaller than the peak dose, while average alveolar region doses (generations 20-23) are on the order of a 100-fold less.

The morphometric data utilized to obtain tissue dose curves in the lungs of rats, guinea pigs, and rabbits (Kliment, 1973) are classified according to "morphometric zone" rather than airway generation so that several human airway generations may correspond to a morphometric zone in these animal species. Predicted tissue doses of NO_2 in the lungs of these animal species are shown in Figure 8. The shape of the dose curves for guinea pigs and rabbits appear to be more similar to each other than to the dose curve for rats. It is interesting that the predicted dose curve for rats is relatively flat over major regions of the tracheobronchial tree. Also, alveolar ducts are predicted to be the region for the maximal tissue dose of NO_2 in rats, whereas the maximal dose region is the last generation of bronchioles in guinea pigs and rabbits. This shift is probably reflective of the model formulation concerning the transition from mucous lined to surfactant lined regions of the lung and of the lack of respiratory bronchioles in the rat. Unlike the more than 100-fold difference seen in man between the peak dose and the dose in more distal regions, a similar comparison for the dose curves in the animal lungs indicates only about a 10-fold difference.

SPECIES SENSITIVITY

Even when the dosimetry is known, the biological response to a given dose may be different in various species. An accurate extrapolation must, therefore, take such potential differences into account. When accounting for species differences, the simplest possibility is when the mechanism of action is common among species, but input factors or repair factors affecting the magnitude of response have graded levels across species.

For a given pollutant the ultimate mechanism of toxicity should be similar across species. For example, with O_3, the initiation of the reaction of O_3 with unsaturated fatty acids (such as are found in cell membranes) involves a direct attack of O_3 on the carbon-carbon double bonds of the fatty acid (Menzel, 1976). While these fatty acids and their double bonds are identical in all species, there are species differences in the proportion of classes of these unsaturated fatty acids. In addition, this initial O_3 reaction results in the formation of reactive intermediaries. These steps are influenced by such things as the concentrations of other chemicals (like levels of vitamin E or antioxidant enzymes) which also differ across species. In addition, repair mechanisms which have a great influence on the expression of toxicity differ across species. These factors ought not to override the basic similarities. For example, the fact remains that when monkeys, rats, cats,

or mice are exposed to low levels of O_3, there is a sloughing of Type 1 alveolar lining cells (across which gas exchange occurs) and a replacement by Type II cells which are thicker and perform another function in the lung (Boatman et al., 1974; Dungworth, 1976 Evans et al., 1976; Mellick et al., 1977; Schwartz et al., 1976; Stephens et al., 1974). For this type of effect, the sensitivity issue relates to the tissue dose-response characteristics and related mechanisms across species so that the human response can be predicted quantitatively.

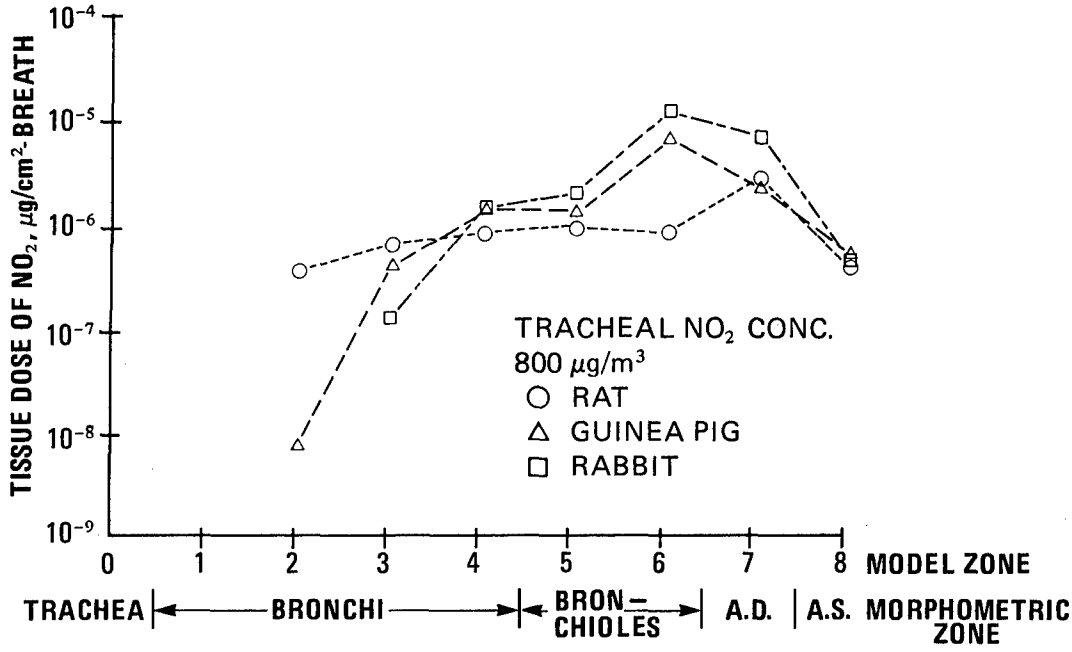

Figure 8. Predicted NO_2 tissue dose curves in the lungs of rats, guinea pigs, and rabbits for various morphometric regions for a tracheal NO_2 concentration of 800 $\mu g/m^3$. For rats, guinea pigs, and rabbits, tidal volumes of 0.95, 1.8, and 21 ml and breathing frequencies of 105, 90, and 50 breaths/min., respectively, were used. Alveolar ducts and sacs were represented by A.D. and A.S., respectively.

FUTURE RESEARCH NEEDS

Research is needed in dosimetry, sensitivity, and health effects to meet the ultimate goals of extrapolation modeling. Respiratory tract morphometric data are vital for dosimetry models of both gases and particles. The nature and structure of the respiratory tract in man and animals critically influences the deposition of both particles and gases since the relative contributions of the mechanisms affecting particle deposition and of gas transport processes vary as a result of this morphology (Bohning et al., 1975;

Pattle, 1961; Taulbee and Yu, 1975; Wilson and Lin, 1970; Yu, 1975). The human tracheobronchial tree is more symmetric, with respect to diameter ratios and branching angles, than that of dogs, rats, or hamsters, but is closest to that of the dog (Phalen et al., 1978). The structure of the tracheobronchial tree is variable from species to species, from lobe to lobe within a given lung, and from one depth to another in the lung. Detailed morphometric data are available for some animal species (Kliment, 1973; Phalen et al., 1978; Raabe et al., 1976) as well as for the normal adult man (Horsfield and Cumming, 1968; Horsfield et al., 1971; Weibel, 1963), but need to be obtained for other animal species and for potentially susceptible human subpopulations such as children, emphysemics, etc.

The degree to which the tract must be faithful has yet to be determined; however, a comparison of the several respiratory tract models available indicates a wide variation in supposedly equivalent dimensions as well as in structure. An example of this is the types of models used: Weibel's (1963) twenty-four generation based and Landahl's (1950) and Kliment's (1973) eight morphometric zone based models. The appropriateness of comparing the results of simulations using the two types of models is questionable in view of the following.

Table 1 indicates the discrepancy between a "collapsed" Weibel's and Landahl's model for man. Weibel's generations have been lumped to create regions or zones that approximate those of Landahl. In doing so, we have preserved the total number of airways per zone, total volume, cross sectional area, length, and air-tissue interface surface area. The radius of a "created" lumen in a given zone is based on the assumption that it is a right circular cylinder with the length of the accumulated generations and radius commensurable with this length, the total volume, and number of lumen assigned to the given zone.

The values in the table range from less than 0.2 to greater than 6.0. These differences are important since the parameters whose ratios are tabulated are necessary and key inputs for our model. Thus, we not only question whether or not the two respiratory tract models (either in zone or generation form) represent the same lung, but also if either lung model is a valid representation of the human lung. For modeling purposes, improved data are needed to define not only the structure or geometry of the respiratory tract but also the dimensions of the needed parameters.

The airway luminal gaseous pollutant concentration at a given level of the lung reflects the dose at that level. Prior to striking the airway wall, the pollutant must first penetrate the mucous or surfactant layer lining the airway, depending on which region of the lung the gas has reached. Chemical reactions may occur in these layers which increase the total pollutant absorbed; however, the amount of pollutant reaching the tissue may be reduced. Thus, the

extent to which mathematical models can incorporate chemical reactions is a function of how much is known about 1) the biochemical composition, turnover and synthesis rates in the mucous and surfactant layers, and 2) the nature of the reactions of the pollutant with the components of these layers.

TABLE 1. A COMPARISON OF LUNG MODELS BY LANDAHL AND WEIBEL

Zone	REGION	GENERATIONS	$\frac{N_L}{NC}$	$\frac{V_L}{V_C}$	$\frac{A_L}{A_C}$ (1)	$\frac{1_L}{1_C}$ (2)	$\frac{S_L}{S_C}$ (3)	$\frac{R_L}{R_C}$ (4)
0	Trachea	0	1.0	0.8	0.8	.92	0.8	0.9
1	Bronchi I	1	1.0	0.9	0.7	1.4	1.1	0.8
2	Bronchi II	2	3.0	1.0	0.7	1.6	2.3	0.5
3	Bronchi III	3-6	0.8	0.4	1.0	0.4	0.7	1.1
4	Bronchi IV	7-11	0.2	0.3	1.4	0.2	0.2	2.7
5	Bronchioles I	12-16	0.4	0.5	2.2	0.3	0.7	2.1
6	Bronchioles II	17-19	0.2	0.2	0.6	0.4	0.2	1.8
7	Ducts	20-22	3.5	0.2	2.4	0.1	0.5	0.8
8	Sacs	23	6.2	1.2	3.1	1.0	1.2	0.7

L = Landahl as cited in (1950); C = Weibel's model "A" collapsed to regions (1963), N = Number of airways, V = total volume, A = total cross sectional area, 1 = length of region, S = surface area, R = radius of airway.

(1) $A_C \sum V_C/1_C$. (2) 1_C = Accumulative sum of airway length in a region. (3) $S_L \quad 2\pi R_L \cdot 1_L \cdot N_L$, S_C = Accumulative surface area of region. (4) $R_C \sum \sqrt{A_C/(\pi \cdot N_C)}$.

A general model of gaseous deposition incorporating a structure for considering chemical reactions and the factors influencing gas transport utilizing the best available morphometric, physiologic, and physicochemical data has been developed (Miller et al., 1978). The more accurate the information on the various components comprising the model, the more quantitative the dosimetric estimate will be. Consequently, research in a number of areas is critically needed.

In estimating pollutant concentrations responsible for observed pulmonary effects, nasopharyngeal removal of the pollutant plays an important role. Experimental estimates of nasopharyngeal removal serve to determine appropriate starting concentrations when modeling pollutant gas transport in the lower airways. Previously, upper airway studies on ozone (Miller et al., 1969; Moorman et al., 1973; Vaughan et al., 1969; Yokoyama and Frank, 1972), sulfur dioxide (Brain, 1970; Corn et al., 1976; Dalhamn and Strandberg, 1961; Fry and Black, 1973; Yokoyama, 1968), and nitrogen dioxide (Corn et al., 1976; Yokoyama, 1968) have been conducted. However, except for SO_2, little is known about the upper airway removal in man of these gaseous pollutants. Nasopharyngeal uptake during normal respiration versus exercise and for different breathing routes needs to be studied.

While the effective axial diffusivity in the lungs of man as a function of the mean axial gas velocity has been studied (Scherer et al., 1972), this work needs to be extended to animals. The vorticies imparted by the bifurcation angles and branching patterns in the lungs of laboratory animals may result in significantly altered diffusion coefficients in the conducting airways. This could change the dose curves for the tracheobronchial region, but it is not likely to influence pulmonary dose patterns, since molecular diffusion is the dominant mechanism facilitating gas exchange in the pulmonary region.

The thickness of the mucous layer in rabbits (Luchtel, 1976) has been used as the basis for assigning thickness values in other species that have been modeled. Except for the rat (Luchtel, 1978), studies on mucous thickness in man and in other laboratory animals are not available and are needed before more definitive comparisons of conducting airway gaseous doses can be made. Also, rates of production of mucus in various parts of the conducting airways need to be established. Experimental data are needed to judge the validity of assumptions about the average rate of production of mucous reaction components being the same for various species.

The diffusion coefficient of various gaseous pollutants in mucus and surfactant needs to be established. Since the diffusion coefficient is important in establishing the mass flow rate of the gaseous pollutant from the lumen into the mucus and surfactant layer, better estimates of the diffusion coefficients would allow a more accurate prediction of absolute differences in dose between species.

To provide a dosimetric model for particles, data are needed in a number of areas. Regional deposition of coarse mode particles in normal adult man and in most animal species has not been thoroughly studied and significant improvement in knowledge of fine particle regional deposition in animals is also needed. To meet the mandate of the Clean Air Act to protect potentially susceptible subpopulations, deposition data for such populations are required. These

populations would include children, individuals with chronic obstructive pulmonary disease, etc. Ostensibly, individuals may be at a greater risk while exercising due to increased ventilation and consequently an increased dose and/or a different pattern of regional deposition. Some of these latter changes may reflect the switch from nasal to oronasal breathing with increased ventilatory demand. The influence of the varying degrees of oronasal breathing on deposition must be studied if the currently available data from experiments using mouthpiece breathing are to be fully utilized.

In determining the species sensitivity, it is necessary to deliver a known dose to the cells/tissue of several species and measure a variety of biologic responses. Major categories might include host defense mechanisms against infectious disease, pulmonary physiology, lung biochemistry/pharmacology, and pulmonary morphology. The dose could be delivered in two ways: 1) in vitro so the actual dose is known, and 2) in vivo (inhalation) in cases where dosimetry estimates are available and where the measurement is not amenable to an in vitro approach (i.e. pulmonary physiology).

When extrapolation models are available, they must be applied to the existing data base. The currently available data base consists of mostly scientifically sound studies conducted in various laboratories with different techniques. The problem can be visualized by examining the available information on the health effects of NO_2 and O_3. There are almost no series of studies which permit species comparisons since several different species were used. There was no commonality of sex and age of the animal, concentration and length of pollutant exposure, or measurement techniques. The above situation will not be resolved by simply increasing funding for any health effect research. The entire data base for NAAQS pollutants must be critically reviewed for gaps relevant to extrapolation models. For each pollutant, such a search might reveal various health endpoints for which increased knowledge would directly aid in extrapolation.

Although the emphasis of the above discussion has been on quantitative extrapolation, qualitative aspects cannot be ignored. These latter aspects relate to improving knowledge of the mechanisms of toxicity for the most significant health effects. Much information is available on these mechanisms, so again the issue is refinement of the data base by highly goal-oriented studies. Knowledge of mechanisms is the cement that binds together the various elements of quantitative extrapolation.

SUMMARY

Environmental toxicologists are confronted with the formidable task of interpreting the results from epidemiological, human clinical, and animal studies on air pollutants and assessing their relevance and implications concerning pollutant levels to which man can

be safely exposed. Usually, the fraction of the total available toxicologic data base represented by epidemiologic and human clinical studies is quite small compared to the data available from animal studies. This data imbalance stems from the nature and inherent limitations of epidemiological and human clinical studies. By contrast, animal experimentation provides the choice of a wide range of concentrations, exposure regimens, chemical agents, biological parameters, and animal species. Relating dose response results obtained in animal studies to the human experience is currently difficult. Many of the these considerations can be resolved through the development and use of pulmonary extrapolation models which account for species differences in dosimetry and tissue sensitivity to the pollutant and the selective improvement of the health effects data base.

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Paper 15

COMPARATIVE MORPHOLOGICAL RESPONSES TO INHALED TOXICANTS

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Text of this presentation is not available for publication.

OPEN FORUM III

DR. LUBET (Microbiological Associates): Dr. Last, you showed data about proline incorporation into collagen after ozone exposure. Do you also get this effect from paraquat and from bleomycin?

DR. LAST (University of California, Davis): Yes.

DR. LUBET: Dr. Brain, you showed the effects of shallow breathing on the distribution of technetium in the dog. Would you expect similar effects if you went to smaller animals? What do you feel the relative problems are when you're exposing animals to aerosols between whole body and head only exposures? In whole body exposures isn't it important that by preening they're taking in a great deal of the dose by the intragastric route?

DR. BRAIN (Harvard University School of Public Health): The first question was, "Is the breathing pattern important in small animals as well as in the dog?" We have less data for small animals but I think the answer is clearly yes and the answer depends on how shallow and how rapid. I believe the same thing is clearly true for man if you want to put aerosols in your upper airway, if you just pant rapidly and shallowly with the tidal volume less than the dead space you can selectively put particles into the large airways. One exception to that is high frequency ventilation when you go to very high frequencies which is known to adequately support metabolism, produce adequate gas exchange, and is associated with aerosol transport into the deeper lung. I'm sure the same kind of changes occur in man. The other question, I think, is a very important one in that it related to the role of preening. I think this is very important and there's not a lot in the literature although I know a number of people have looked at it including ourselves. The question is what's the difference between a head only exposure with some of its problems such as altered breathing pattern and putting in a free ranging animal who is going to get particles deposited on his fur. Most small animals do a lot of preening of themselves and of their cage mates. We have found that about as much material in the whole animal could come from preening as by breathing. So if the material is one that dissolves in the GI tract and is absorbed and goes to other organs, deposition on the fur and preening could be very important. There are a couple of ways to get around the problem. One is to use head only exposure and the other way is to try and clean the animals off. For some kind of experiments, we have tried to wash the animals off immediately after a brief exposure and dry them with hairdryers. That can markedly reduce the amount of material that gets into the GI tract. Depending upon the

agent, its solubility, its fate, its action in other organs, one certainly has to be alert to the importance of preening behavior. It can be very important.

DR. LAST (University of California, Davis): In the same light we also ought to realize that while we use phrases like detoxification for the process of trapping things in the mucous layer, mucociliary clearance actually ends up with swallowing the material that is cleared from the lung. Even if we deposit the aerosol into the lung, it may ultimately end up being administered intragastrically as well. It isn't clear to me that we necessarily ought to feel good about the fact that the ozone reacts with surfactant or reacts with the mucous; all that we do is put another set of reactive compounds into a different portal of entry and end up getting exposed to those products down the line. I think there's a lot of judgment in using the term detoxification for the lung clearance process that may not be justified.

DR. MILLER (United States Environmental Protection Agency): I'd like to respond to that because I think you're right. There is also reabsorption of fluid as you go up the respiratory tract so it is not a simplification to say that it's a detoxification process because the whole role of interactive and reactive products is certainly not well studied. In terms of gradients of response, when you look at the histopathologic data you certainly find more effects at lower concentrations deeper in the lung, but as you go up in concentration you also get effects in higher airways. It is certainly a concentration dependent phenomenon.

DR. CAVENDER (The Mitre Corporation): I'd like to ask Dr. Tyler to elaborate on the time response for the induction of new respiratory bronchioles in the rodent. Do those go away with time? If so, what is the time response?

DR. TYLER (University of California, Davis): The induction of respiratory bronchioles started to occur at a little more than 50 days, somewhere between 50 and 90 days, and they persisted and became more noticeable until 180 days when the exposure ended. In the postexposure period, they did tend to regress some. I wouldn't think they would regress all the way because there should be a fair amount of fibrosis around those by then. They probably will never regress totally. We're looking at that point now in another study in which we'll make plastic casts of the airways to look at airway generation with that method.

DR. LAWRENCE (Albany Medical College): Dr. Brain, in your presentation you suggested that there are some species differences in bacteriocidal killing mechanisms. Could you elaborate on that?

DR. BRAIN (Harvard University School of Public Health): One place to look is a review of bacteriocidal mechanisms by Gary Huber in a book edited by Reed, Proctor and Brain called, Respiratory Defense Mechanisms. Huber goes into some detail about the killing of different organisms and it is apparent from looking at that chapter that there are a lot of species differences. There is also a recent paper published that reported big differences in phagocytosis and killing by macrophages among humans, rabbits, rats, and hamsters.

DR. LAWRENCE: I was wondering about what you had said in your presentation because you were talking about phagocytic capabilities but some were bacteriocidal actions and those things are really different. You're talking about the actual killing mechanisms versus the ability to take up these different pathogens.

DR. BRAIN: Yes, I think there are probably differences at every stage. I presented our own data for colloidal gold where there are differences in the rate of phagocytosis of that inert material. I think there are differences in relation to the importance of opsonins in binding and ingestion, and finally I'm sure there are also differences in the kind of bacteriocidal mechanisms that occur intracellularly. I think they all need to be explored and we need to search for not only differences but for why they occur so that we will have more predictive power. This area of research, as far as I'm concerned, seems to be in its infancy, lacking systematic coherent descriptions of species differences.

DR. COURI (Ohio State University School of Medicine): Dr. Brain, the proposition I share with you is that it is difficult to express particulate exposure in animal systems. Do you have another index you might want to suggest? Or is there a way where one can evaluate those relationships? Do you have an idea for an alternate expression of exposure to those?

DR. BRAIN (Harvard University School of Public Health): Whenever possible, people ought to look for the material. If they're giving particles of various kinds, rather than just measuring the chamber concentrations, they ought to sacrifice some animals and if it's a material that can be detected in the respiratory tract, they ought to measure its distribution in the respiratory tract. If that's not possible, then perhaps the use of some tagged material, fluorescent or radioactive, such as technetium 99 which has a six-hour half life. It seems to me, as much as possible, we should try to estimate the amount and distribution of dose. We can, for example, look at the disappearance of inhaled material if you can manage to look at the inspired and expired concentrations, at least

you can estimate what's different. You can use some of the modelling approaches and at least be aware that smaller animals do breathe more per gram body weight than larger ones. I think you're absolutely right. It is certainly much easier and straight-forward to measure the chamber concentration and not worry about individual variability among animals. Even if you only measure chamber concentration you should be aware that it's not measuring what's in the animals and that ought to be clear from your paper and clear in your own thinking that you've only measured what's in the air and not what's in the animals.

DR. COURI: Well, even with the suggestions you've made, one can argue that they simply represent clearance, that is uptake by certain cells or deposition which may represent sequestration and not really the site at which these agents are activants. But I think that's sufficient. Thank you. I'd like to ask Dr. Last a question in regard to the labeling of glycoproteins with the in vitro system. How do you look upon that as an index of glycoproteins, in that glucosamine is generally the first sugar put onto a polypeptide backbone? Aren't you really measuring glucosamine transferase activity rather than glycoprotein synthesis in that case? The second part of my question is would you compare sialic acid incorporation relative to glucosamine incorporation that gives you the glycoprotein synthesis mechanism?

DR. LAST (University of California, Davis): The material we looked at has been secreted into the medium and therefore, theoretically at least, is complete even though the glucosamine may have gone on anywhere. Secondly, glucosamine in these systems and in most crude cellular systems is a precursor of sialic acid itself and a fairly efficient one. It turns out to be fiendishly difficult to do isotope incorporation specifically to look at sialic acid. Where we treat the material with neuraminidase to ask the question how much of the label is in sialic acid in these molecules, we find it's about 5 or 10% at the low end of the extreme and perhaps as much as 20 to 30% on the high end depending on the way we do the experiment. And as you suggest, if we do a very short-term labeling, we tend to see less of the label in the interior glucosamine and more in the terminal units of the glycoprotein.

DR. WHITTENBERGER (University of California, Irvine Southern Occupational Health Center): I have a question for Dr. Mauderley. I was struck by the contrast between his presentation and Dr. Brain's. Dr. Brain seemed to emphasize differences among human respiratory responses and the respiratory responses of other animal species, whereas Dr. Mauderley seemed to emphasize similarities between human respiratory responses and those of other animals. I

don't believe that there really is that much discrepancy between their points of view but I'd like to hear both of them comment on that subject.

DR. MAUDERLEY (Inhalation Toxicology Research Institute): I don't believe that there is much difference either. Perhaps I'm taking the role of the optimist here; those of us that make our living extrapolating or trying to extrapolate these functional changes would like to believe that there are no differences. I think that what Dr. Brain and I are trying to do is a bit different. What I was trying to do was to provide some instances of data sets that you can use to make direct comparisons between animals and man. There are not many data available for this purpose. Differences in exposure, materials, dosimetry, and methodology make comparisons somewhat difficult. I think that there is not much of a discrepancy and I think in terms of a functional response as opposed to a cellular response that if you induce the same type of injury or the same morphologic change in various species, you will see a similar functional response measured physiologically. That does not mean if you treat animals and man similarly that you will induce the same morphologic changes and that's wherein the difference lies. What I was trying to illustrate is that in cases where you can induce similar morphologic changes, the functional manifestations will be similar.

DR. BRAIN (Harvard University School of Public Health): I think I agree with that. I think your real question was are there good animal models of human lung injury? I think the answer to that is yes, there are some that have the same biochemical, morphologic, and physiologic hallmarks of human lung injury. A related question is, are there good animal models of human lung disease? Again, I think the answer is yes. They have improved a lot and they're getting better all the time. On the other hand, I think those questions are distinct from trying to use animal models to establish dose response relationships and trying to find dose levels that are safe. I think there the problems of dosimetry become more troublesome. Again, they vary. If you take radiation, oxygen, or carbon monoxide, the problems are relatively simple because the dose per gram of lung is probably pretty constant and doesn't vary very much as a function of animal species. On the other hand, with formaldehyde or SO_2, which are very water soluble, you're likely to have very different distributions of dose as a function of animal species. I would ask a question of Dr. Miller, which I think maybe will illustrate some of the difficulties in making these kinds of comparisons. Dr. Miller, you showed us a couple of slides of the burden ratio which tried to compare the lung burden in rats and man and again, I think that it was reassuring that they were fairly similar. I believe there was also data from Raabe on the slide. As you may remember, the dose, the burden ratio varied from, I think, 0.2 to 1.6 and for one micron particles it was 1. On the other

hand, it was expressed in terms of particles per unit area. Is that an appropriate way of comparing dose? I would remind you that in rats the alevolar area per gram of lung tissue is really very different from man. Rat alveoli are smaller and thinner and there are more alveoli per gram or more surface area. If you express the dose in terms of particles per gram lung, then you'd find that you had a much higher dose in the rats than you did in humans. Cells are about the same size in rats and humans so if you really wanted the dose per cell, then I think you'd see a much larger difference. Is that an appropriate denominator, particles per surface area? Why not per gram lung or per cell?

DR. MILLER (United States Environmental Protection Agency): Part of the response there is for insoluble particles and this brings up an interesting point with respect to the surface area estimates that are available. We have adjusted these to a functional surface area as opposed to the morphologic available surface areas. When you make that conversion you take into account some of the differences that you're talking about. If the particle is soluble, there is a difference. When you apply the kinetics of the transfer of the particle to various regions, you can have different expressions. I would agree that, depending upon the particular compound and the endpoint you're looking at, there are different ways that you may wish to express the dose that will relate better to the biological effect. I didn't have time to go into that, but in answer to the first part of your question, these data are information that is available in the literature, the human data are taken from the EPA SO_x document that I hear will be released by the end of the year. The animal data that were used are from Otto Rabbe's publications and it does make a difference depending upon the kind of particle.

DR. WHITTENBERGER (University of California, Irvine Southern Occupational Health Center): I had a related question for Dr. Brain and also I'd like Dr. Miller to comment. I was struck by Dr. Brain's emphasis on the lack of standardization of exposure conditions in experimental protocols of different species. I wondered if the comprehensive analysis of the species difference wouldn't be promoted by voluntary cooperation among inhalation toxicologists. When some similar problems were addressed in the field of controlled human exposures to air pollutants, an agreement was reached by the research experts in this field that they would have a regular conference at which they would standardize protocols. Would that be a worthwhile consideration? I realize that the problems of human exposure are very different from the problems of experimental animal procedures. It seems to me there might be benefits from a cooperative effort.

DR. BRAIN (Harvard University School of Public Health): I think that sounds like a good idea. In addition to species

differences which we've emphasized, there are also differences that relate to age, to the kind of restraint used during the exposure, the temperature, which again is not always carefully controlled, the amount of ventilation and the breathing pattern is also a function of chamber temperature. So I think there are a host of things that it would be good to get together and talk about.

DR. MILLER (United States Environmental Protection Agency): Let me follow up on that subject. While the idea is great, the complexity of the problem probably says that there is not a practical solution; on the other hand, extremely good characterization in Journal publications of your exposure system will go a long way toward being able to understand some of the factors that contributed to the differences in response. Given the type of biological input that may be under investigation in an animal study, it may not be practical to have massive coordination. Bear in mind some of the situations that you have to look for in regulatory efforts. It has gotten to the point that if you don't state in your paper the mass median aerodynamic diameter of the aerosol and its standard deviation as well as some other factors, that paper will not even be considered for inclusion in a criteria document. A lot of toxicologic studies will have gone for naught. In litigation actions, it's very important to be detailed on the procedures that were used and while oftentime Journal reviewers want you to cut down on this information, if it doesn't appear in the written word, the regulatory usefulness of it is greatly reduced.

DR. COURI (Ohio State University School of Medicine): I'd like to comment on this business of science by consensus or regulation by a select few. I think that a scientist doing an experiment deserves to express the data he thinks best exemplifies the hypothesis being tested or examines that hypothesis. I believe it was Dr. Brain who just made the comment which I think is appropriate, if you are dealing with a particle that's emitting radiation, there's one way to express that to get the meaning of that experiment across which may be totally different from something that a consensus committee comes up with for how to express data dealing with particulates in lung - whether it be body mass or lung mass or surface area as such. I would like to leave science wide open to free expression. We are having this particular problem in my laboratory at this time. We look at hyperplastic islet cell foci in liver after chemical stimulation and there is a trend now to express this change as islet cells per centimeter cubed as opposed to islet cells per centimeter squared. The important thing is that the experimental data are not different whether you say cubed or squared but you have a $100,000 instrument to measure the cubic volume as opposed to a $20,000 instrument to measure area. That's a problem. What it does is exclude good science, simply on the basis of some ultrasophistication in measurements. I think we ought to leave room for scientists to at least get their data into the literature to be

examined and if one wants to recalculate whether or not some particle relative to lung area is also relative to body area, that's the prerogative of the reader. I think one must leave room for creative science.

DR. WHITTENBERGER (University of California, Irvine Southern Occupational Health Center): I'm certainly sympathetic to that point of view and I'm sure the whole audience is. Obviously, the creativity and originality of individual scientists is very much to be preserved.

DR. COURI: Thank you, but unfortunately, those people making those consensus are also editors of Journals, also people who will lead discussions of this type, people who are in meetings, who arrange symposia and such and if you're not one who wants to dedicate that kind of time to that activity but rather be in a laboratory doing experiments, you are not privy to those consensus opinions.

DR. MAUDERLEY (Inhalation Toxicology Research Institute): I think that what we're talking about is not setting up a set of standard exposure atmospheres or concentrations to which all people have to expose animals to conduct experiments, but rather the plea that I hear Dr. Miller making and I would certainly echo, which is to provide complete information in the publication. Anyone who has gone through the literature trying to make extrapolations or trying to abstract information on dose response relationships, even qualitative such as the very few comparisons that I've made today, will find this is a really difficult if not impossible task if there are missing numbers in the paper. The numbers that need to be there if you're dealing with an inhaled particle need to reflect not only the air concentration of those particles but also particle sizes. We know that there can be marked differences in the acute responses to materials such as sulfuric acid mist depending on whether the particle size is around three tenths micron as opposed to one micron and that's not a big difference in particle size. For instance, the acute lethality of that difference in H_2SO_4 particle size in some animal models are orders of magnitude different. What I'm hearing and what I would certainly echo is a need for people to conduct their experiments as they see fit but to be careful to characterize those exposures and express the data in such a way that Dr. Miller from his viewpoint and I from my viewpoint and other people who are interested in certain types of effects or certain types of extrapolations can perform those calculations. Without the data in the paper, it is impossible to perform the calculations.

DR. MILLER (United States Environmental Protection Agency): I'd like to follow up on that. I think the plea is certainly not for science by committee. We are for good science individually but

with documentation and the plea is probably against editors and reviewers of manuscripts who want a lot of that material cut out that they don't see as contributing to the discussion of the results. I don't think we're at odds on the opinion of the appropriateness of the individual scientist stating up his hypotheses and the extent to which he expresses his data. I'd like to ask Dr. Mauderley a question. I'm wondering if he would care to comment on the uniqueness of the guinea pig as a model? Do you think that the guinea pig might be a unique animal model appropriate for comparison with human effects?

DR. MAUDERLEY (Inhalation Toxicology Research Institute): I think that anyone who has examined the subject and who has worked with guinea pigs recognizes that among the more common laboratory animal species they are sort of an outlyer in several respects. They are strange animals. I think that in determining whether or not this is an appropriate animal species again depends upon the type of effect that you are trying to measure. If you are interested in acute bronchiole constrictive responses that occur, then I, as most people who have worked in this field, believe that the guinea pig is probably an appropriate subject because it does seem to have an airway reactivity that is quantitatively more similar to that of man than does the rat to many kinds of materials. Again, it depends upon the endpoint, it depends upon the reason for doing the study. In any experimental design you first have to decide what you're going to try to study, and then you select an experimental animal model that will fit your needs. Guinea pigs really have not been used in a lot of the chronic inhalation studies with various pollutant materials. There really are not enough data to define the appropriateness of the guinea pig in many situations. If you are looking at acute airway restrictive events, or broncho-constrictive events, or if you are looking at alterations of airway sensitivity then it would appear to be an appropriate model for man. I think the simple comparison that I made this morning leans in that direction and you can compare other data too. It's my plea to not start, as some of us want to do, with our favorite animal and our favorite endpoint and go looking for a study to do. But rather determine what studies need to be done, and then pick the endpoints and the animals that are appropriate to examine that question. I'm not sure that's an answer to your question, but that's the answer I have to offer.

DR. WHITTENBERGER (University of California, Irvine Southern Occupational Health Center): Dr. Tyler, do you have any comments on this issue?

DR. TYLER (University of California, Davis): Yes, I think selecting the experimental animal is one of the major factors in research. If you go back and read Claude Bernard, the founder of

experimental medicine, you will find that he states very clearly that the selection of the animal upon which to experiment is going to make a major difference in your results. I think this is really a very critical thing. The guinea pig is indeed a wonderful animal for some of these responses. However, its lungs contract so much that the airways are closed down and it is very difficult to get fixation by the conventional means which allow you to do morphometric studies such as those done by Weibel or my colleague Hyde. The selection of animal species is extremely critical if you're going to apply more than one method of examining the lungs. I showed the slide of all the various clara cells that's published in Experimental Lung Research, either in Volume I or II. The important thing here is that the enzymes in clara cells most likely vary with structure. If you're interested in something which is going to be metabolized by P450 and if you don't use an animal whose clara cells have a fair amount of agranular or smooth endoplasmic reticulum, you will have a low amount of P450 and you're going to get different results than if you use an animal species with large amounts of agranular endoplasmic reticulum in its clara cells. There are some very significant species differences which influence results. I think some of the tests which have been conducted using hamster trachea are going to be subject to question because of this.

DR. WHITTENBERGER: Dr. Tyler, does the guinea pig have smooth muscle in the visceral pleura which responds similarly to that of the bronchiolar muscle?

DR. TYLER: I really don't know. I haven't looked at the pleura of that animal. The lung contracts tightly and it's extremely difficult to fix satisfactorily. Dr. Brain, you and your colleagues at Harvard use guinea pigs in many studies. Have you solved this problem?

DR. BRAIN (Harvard University School of Public Health): I don't know either! I've had the same experience. It's very hard to do lung lavage in guinea pigs because the lung constricts so tightly you can force the fluid in but you can't withdraw it. Putting isoprel in the lavage fluid keeps the airways open and you can lavage the lungs in that manner.

DR. ALI (Ohio State University School of Medicine): Dr. Tyler, you mentioned that there is a destructive phase upon exposure to ozone. I would like to know if this destructive phase is actual necrosis of the cells and whether this event occurs preceding the hyperplasia. Is the local area of the destructive phase replaced by fibrosis or by hyperplasia?

DR. TYLER (University of California, Davis): I think the best information available at the present time is in a paper by Dr. Castleman, who is the senior author, in the American Journal of Pathology. I think that's the most quantitative study and probably the best. The times for various events were more precise than most studies. It involved monkeys so there were respiratory bronchioles as you saw. The damage to the type I cells and to the few ciliated cells in the respiratory bronchiole occurred early and the damage was very noticeable. There was necrosis and Dr. Castleman has counted the number of necrotic cells. While I say this is well quantitated Dr. Castleman did not take into consideration the three dimensional structure of the cells, rather it's in two dimensional study units, but nonetheless, it is reproducible. We've done some more experiments since then and we get about the same results. The destruction of ciliated cells continues but the type I hyperplastic cells are resistant to ozone. Cells derived from the type I cells that have not aged very long are also relatively resistant. They are a primitive cell and have a lot more organelles indicating they'd be resistant. These tracks of ciliary destruction are seen in chronic exposures as well as in acute exposures. The site of the cytofibrosis that I have seen in monkeys in some of my own studies has been predominantly on the back side of respiratory bronchioles. Dr. Last did some studies with Dr. Castleman in rats. What were the results there, please?

DR. LAST (University of California, Davis): With rats, the fibrosis is predominantly in the alevolar duct. There is an interstitial component as well so that it probably follows the destruction of the type I cells.

DR. TYLER: Was there any correlation with macrophages?

DR. LAST: The only good data I have seen on that point didn't come from our ozone work, albeit we've been trying to do that. Dr. Peter Witche has done a lot of work with the BHT oxygen model and in that case there is a correlation between where the macrophages accumulate and seem to occur in greatest numbers. I believe that there would be a correlation with the macrophage but we haven't done that study carefully enough to know that in the ozone model yet.

DR. CROCKER (University of California, Irvine): I've been impressed by the degree to which we've been looking for converging evidence and data and certainly that is more and more the case for ozone and NO_2, for example, where solubility in the upper airways is not as important and yet we do see very comparable lesions in animals of varying complexity and anatomic structures in the distal airways. I think that the concerns that many of us are now having

when we see exposures to a variety of pollutants in ordinary environmental circumstances or in occupational settings are that there are degrees of solubility in the upper airways such that it will make difficult the comparison between the species because of the complexity of the nasal turbinate area and its capacity to absorb. I think that most of Dr. Miller's data relate to the model system beginning at the trachea. I am interested in the question of the degree to which we are absorbing material in the nose. Dr. Miller, would you be willing to make some comment about the comparability or differences in terms of extraction by the nose between species? This is the one area where I think we may find significant species differences whereas in lower airway damage due to ozone or NO_2 we find significant convergence of data.

DR. MILLER (United States Environmental Protection Agency): With respect to ozone and NO_2, there is experimental evidence on extremes for nasal pharyngeal removal of 70 to 95% in dogs down to around 50% in rabbits and guinea pigs. There are some theoretical models. Aaronson has looked at the uptake of soluble vapors in the nose and has looked at SO_2 incorporation.

DR. TYLER (University of California, Davis): There is some very nice work by Schrieder and Rabbe on this subject using casts of the nasal cavity. If you're interested, the results were published in the American Journal of Anatomy about 18 months ago and in this study they did a nice thing. After they made the casts in the usual manner they sliced them and analyzed them morphometrically using the Quantimet image analyzing computer. They measured surface areas and volumes and it would be very possible to break this down into the major air passages, the meatus of the nasal cavity and relate that to the gas disappearance on a single passage through.

DR. BRAIN (Harvard University School of Public Health): I would like to make one other comment. My general impression is that the human nose is less efficient than most animal noses. Dogs, mice, and rabbit noses all seem to be more elaborate and more efficient than the human nose. The other difference is that humans do a lot of mouth breathing. During talking for example, as I've just done now, and certainly during exercise when we increase our ventilation rate by a factor of 2 or 3 we switch from nasal to mouth breathing which certainly doesn't occur in dogs or most other smaller animals. I think that's a very important transition that relates to exercise. Again, it depends on the gas. If you use carbon monoxide which is very insoluble it doesn't make any difference whether it's breathed through the nose or mouth, there's no absorption anyway and exercise just lets you get to an equilibrium carboxyhemoglobin concentration a little faster but the equilibrium is exactly the same. That's in marked contrast to sulfur dioxide or formaldehyde which are very water soluble and are taken up very efficiently.

Drs. Frank, Okayama, and I did some experiments with dogs which illustrate the problem very dramatically. If you pass SO_2 into the nose at 3 1/2 liters per minute and measure it in the trachea just below the larynx you'll find 99.999% was removed. On the other hand, when we switch to mouth breathing and increased the flow by a factor of 10 to 35 liters a minute, then only 70% was absorbed, 30% was still present at the level of the trachea. In this example we've gone from 0.001% to 30%, that's a 30 thousand fold increase in tracheal SO_2 concentration. Now that may be a little non-physiological and it may not be as different as that in man, but at least it illustrates the point. I think the same kind of difference occurs for very large particles. If we're talking sub-micronic particles the nose isn't very efficient, but if we're talking about 10 micron particles, then the nose is a very efficient filter and the mouth very much less effective and so again I wouldn't be surprised to find tracheal deposition of big particles varying by a factor of 10 or 100 as humans switch from nose to mouth breathing. I think the role of the nose is very important, particularly for water soluble gases and large particles.

DR. MILLER (United States Environmental Protection Agency): I think we're trying to point out that it's specific to the given pollutant and the type of particle and that you will be able to make any kind of a generalization. We've currently finished a study with phosgene in looking at distribution in four or five species and we see marked differences in the relative distribution. The interpretation of results is really dependent upon that compound. When you factor in flow rates, solubility, and other factors, there are just not any gross simplifications that you can make.

DR. TYLER (University of California, Davis): When I think of tissue and cell dose, it's a very nice clean concept. I think one of the things though that's important probably is the dose rate. The same tissue dose delivered at a more rapid rate probably has a different effect than that delivered at a slower rate because the cell can simply handle more of it.

DR. MILLER: There is a vast amount of information available for NO_2 on that very point, with endpoints such as the mass infectivity model. When you look at the uptake also from a dosimetric viewpoint and these kinds of things also relate to exercise you see a two and a half fold increase in the flux into the tissues so they very much do relate. There have been human studies, using an exercise regime, that have shown dose rate phenomena. That's why the activity pattern of the population is now something that we have to factor into standard setting because you may find that the current standard for ozone is fine if you're resting but it may not be adequate if you are exercising. Dr. McDonald has just finished a

very detailed study using a heavy exercise comparing dose response with ozone levels and you see these kinds of phenomena. It certainly is a very important factor.

SESSION IV

CONTEMPORARY IDEAS ON MECHANISMS OF TOXICITY OF DIOXIN AND RELATED CHEMICALS

Chairman

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PAPER NO. 16

THE CYTOSOL RECEPTOR FOR 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN: MEDIATOR OF TWO DISTINCTIVE PLEIOTROPIC RESPONSES

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INTRODUCTION

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) serves as the prototype for a large group of halogenated aromatic hydrocarbons (dibenzo-p-dioxins, dibenzofurans, azo(xy)benzenes, and biphenyls) which produce a similar and characteristic pattern of toxic responses, induce a common battery of enzymes involved in drug metabolism, and are thought to act by a common mechanism (Poland et al., 1979; Goldstein, 1980; Poland and Knutson, 1982).

The toxic responses, i.e. histopathologic changes, produced by TCDD and congeners are quite characteristic; however, many of the lesions are highly species specific (Schwetz et al., 1973; Kimbrough, 1974; McConnell, 1980). Following the administration of a lethal dose of TCDD, virtually all species experience a prolonged wasting syndrome prior to death, with loss of adipose tissue, involution of lymphoid organs, degeneration of the seminiferous tubules of the testicle, and embryotoxicity and/or teratogenicity. However, the more distinctive lesions which involve proliferation and/or metaplasia of epithelial tissues such as skin, stomach, intestines, and urinary tract, occur in a limited number of species. Similarly, the presence and severity of hepatic pathology vary considerably among species. Animal species also vary greatly in their sensitivity to TCDD, e.g. the LD_{50} in guinea pigs is 1 μg/kg (Schwetz et al., 1973) and in hamsters, 5000 μg/kg (Henck et al., 1981). The cause of death is unknown.

The most studied of the biochemical responses to the halogenated aromatic hydrocarbons is the induction of hepatic cytochrome P-450-mediated microsomal monooxygenase activities, including aryl hydrocarbon hydroxylase (AHH) activity. These monooxygenase activities metabolize foreign lipophilic chemicals which enter the body to more polar and readily excretable products. Other enzymes involved primarily with drug metabolism, such as UDP-glucuronosyltransferase and DT-diaphorase, are among the coordinately expressed enzyme activities stimulated by TCDD (Poland and Kende, 1977).

Both the histopathologic changes produced by TCDD and the induction of the drug metabolizing enzymes are mediated by a soluble protein species, termed the cytosol receptor (Poland et al., 1976; Poland and Glover, 1980). This is shown by two lines of evidence. First, for a large number of halogenated aromatic hydrocarbons, the binding affinities for this receptor correspond very closely with the rank order for their toxic potencies and their potencies to induce AHH activity (Poland et al., 1979; McConnell et al., 1978; Goldstein, 1980). Second, in mice there is a genetic polymorphism at the Ah locus (Nebert and Gielen, 1972); this locus determines the receptor (Poland et al., 1976). Mice phenotyped as possessing the higher affinity receptor are more sensitive to the production of a biological response by TCDD than are mice with a lower affinity receptor phenotype. Segregation with the Ah locus has been shown for the production of several toxic responses, e.g. thymic involution and hepatic porphyria (Poland and Glover, 1980; Jones and Sweeney, 1980), and the induction of several enzyme activities, e.g., AHH (Poland et al., 1974), and UDP-glucuronosyl transferase (Owens, 1977), segregate with the Ah locus, the locus which determines the receptor.

In virtually all tissues that contain the receptor, TCDD and congeners induce microsomal monooxygenase activity or other enzymes involved in drug metabolism; however, the expression of a histopathologic response is limited, even among those tissues and cells which possess the receptor (Poland et al., 1974; Bradlaw and Casterline, 1979; Knutson and Poland, 1980a). Thus, for the halogenated aromatic hydrocarbons to produce a toxic lesion in a tissue, the presence of the receptor is essential, but not sufficient.

In this report, we discuss two experimental systems as models for TCDD toxicity: a) the response of the epidermis in HRS/J mice and b) the keratinization of mouse teratoma cell line XB. Each system illustrates the two distinctive pleiotropic responses to the halogenated aromatic hydrocarbons controlled by the cytosol receptor: the one primarily concerned with the induction of drug metabolizing enzymes; the other, a more extensive response, involving differentiation. From these data, we suggest a model which may provide a general framework in which to consider the tissue specific and species specific toxicity of the halogenated aromatic hydrocarbons.

EPIDERMAL RESPONSE TO TCDD IN HRS/J MICE

Hairless is a recessive trait in mice controlled by the hr locus on chromosome 14 (Gruneberg, 1952). HRS/J is an inbred strain of mice segregating for the hr locus. The homozygous hr/hr hairless mice and their congenic, heterozygous hr/+ haired littermates are genetically identical except for one allele at the hr locus and other closely linked loci on chromosome 14 (Womack et al., 1977; Green, 1966).

HRS/J mice of both phenotypes, i.e. haired and hairless, have the same affinity and concentration of the cytosol receptor as measured in the liver (hr/hr, K_D = 0.304 ± 0.093 nM, n = 77.2 ± 19.5 fmole/mg cytosol protein; hr/+, K_D = 0.329 ± 0.072 nM, n = 61.8 ± 15.4 fmole/mg cytosol protein). High affinity specific binding of TCDD was also found in the epidermal cytosol of both hr/hr and hr/+ mice. The induction of epidermal AHH activity by TCDD in HRS/J hr/hr and hr/+ mice has a similar time course and dose response curve (Figure 1).

In contrast to the induction of epidermal monooxygenase activity, the histologic response to TCDD differs in the epidermis of haired (hr/+) and hairless (hr/hr) HRS/J mice. Application of TCDD to the dorsal skin of HRS/J mice produces an obvious scaliness and extensive histologic changes in the skin of hr/hr mice, but not hr/+ mice (Figure 2). In HRS/J hr/hr mice, this response, which includes hyperplasia and hyperkeratosis of the interfollicular epidermis, squamous metaplasia of the sebaceous glands, and hyperkeratosis within the dermal cysts, is both time and dose dependent. A total dose of TCDD of 36 pmole produces an equivocal (±) response; 0.36 nmole, a moderate (2+) response; and 3.6 nmole, a moderate to severe (2+ to 3+) response four weeks later. These skin lesions are produced by topical application or intraperitoneal injection of TCDD.

The histologic skin lesion produced in hr/hr mice by TCDD is mediated by the cytosol receptor. For the halogenated aromatic hydrocarbons tested, the structure activity relationship for the production of this toxic response corresponded with that for binding to the cytosol receptor. The production of the skin lesion by a congener of TCDD, 3,3',4,4',5,5'-hexabromobiphenyl, segregated with the Ah locus in mice bearing the trait, hairless (Knutson and Poland, 1982).

The results of several experiments suggest that the haired phenotype, per se, does not account for the different histologic response produced by TCDD in the skin of mice which have or lack hair. First, the rate of removal of ^{3}H-TCDD from the skin of hairless or haired mice which were shaved was similar. Second, application of TCDD to the ear, an area devoid of hair in a haired mouse failed to produce a histologic response. Third, mice bearing mutation naked (N/+) lack hair, but fail to develop epidermal histologic changes from TCDD (Knutson and Poland, 1982).

KERATINIZATION OF MOUSE TERATOMA CELL LINE XB BY TCDD

When XB cells derived from a cloned mouse teratoma cell line isolated and characterized by Rheinwald and Green (1975) are plated at low density ($2x10^2$ cell/60 mm plate) on a feeder layer of lethally irradiated 3T3 cells, XB cells form colonies of stratified, keratinized squamous epithelium, similar to that in epidermis. When XB cells are plated at high density ($3x10^5$) on a feeder

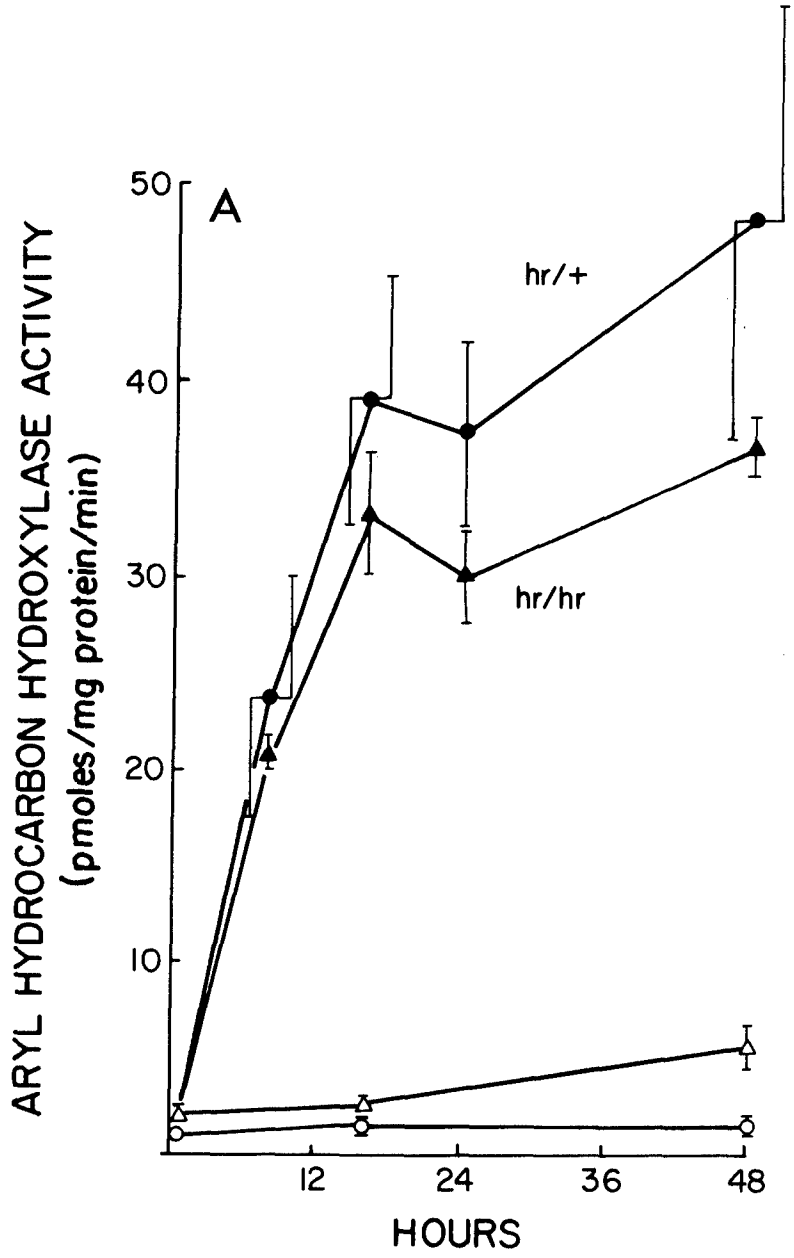

Figure 1. Induction of aryl hydrocarbon hydroxylase activities by TCDD in the epidermis of HRS/J mice.

A) Time course of the induction of aryl hydrocarbon hydroxylase activity in epidermis of 5-7 week old HRS/J hr/hr and hr/+ mice following a single application of TCDD (7.8 pmol). Control mice (open symbols) received only acetone. All animals were killed and the enzyme activity assayed on the same day. Each point is the mean ± standard error of the values of four mice.

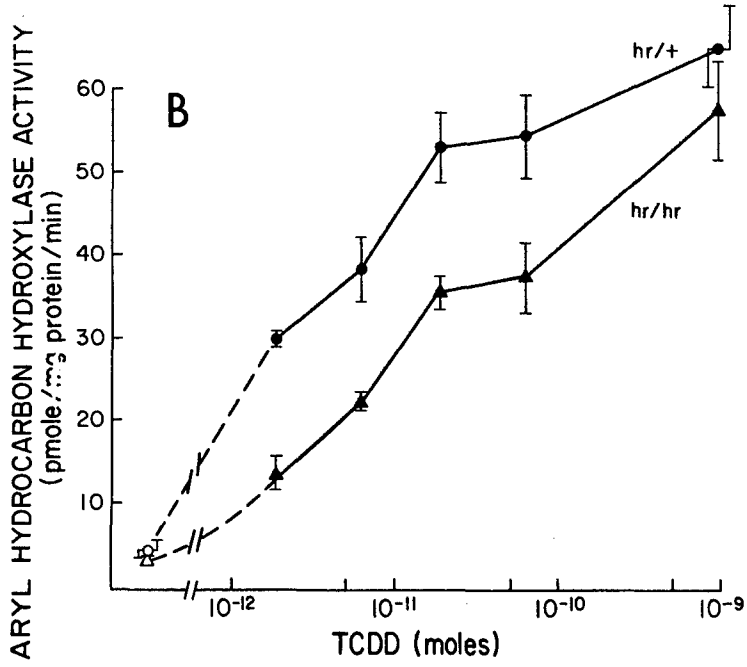

Figure 1. Induction of aryl hydrocarbon hydroxylase activites by TCDD in the epidermis of HRS/J mice.

B) Dose-response curves for the induction of aryl hydrocarbon hydroxylase activity in the epidermis of 4-8 week old HRS/J hr/hr and hr/+ mice 48 hours after TCDD application. The initial point is the value obtained from skin treated only with acetone. Each value represents the mean ± standard error from five mice.

layer of irradiated 3T3 cells, they divide, but do not spontaneously differentiate. Under these conditions of high XB cell density, which prevent spontaneous differentiation, the addition of TCDD produces a dose-related keratinization, with a maximal response at 5×10^{-11}M (Knutson and Poland, 1980b). XB cells contain a cytosol species which binds TCDD with a high affinity, and for over 30 halogenated aromatic hydrocarbon congeners tested, there was an excellent correlation between the potencies of these compounds to produce keratinization and their binding affinities for the cytosol receptor.

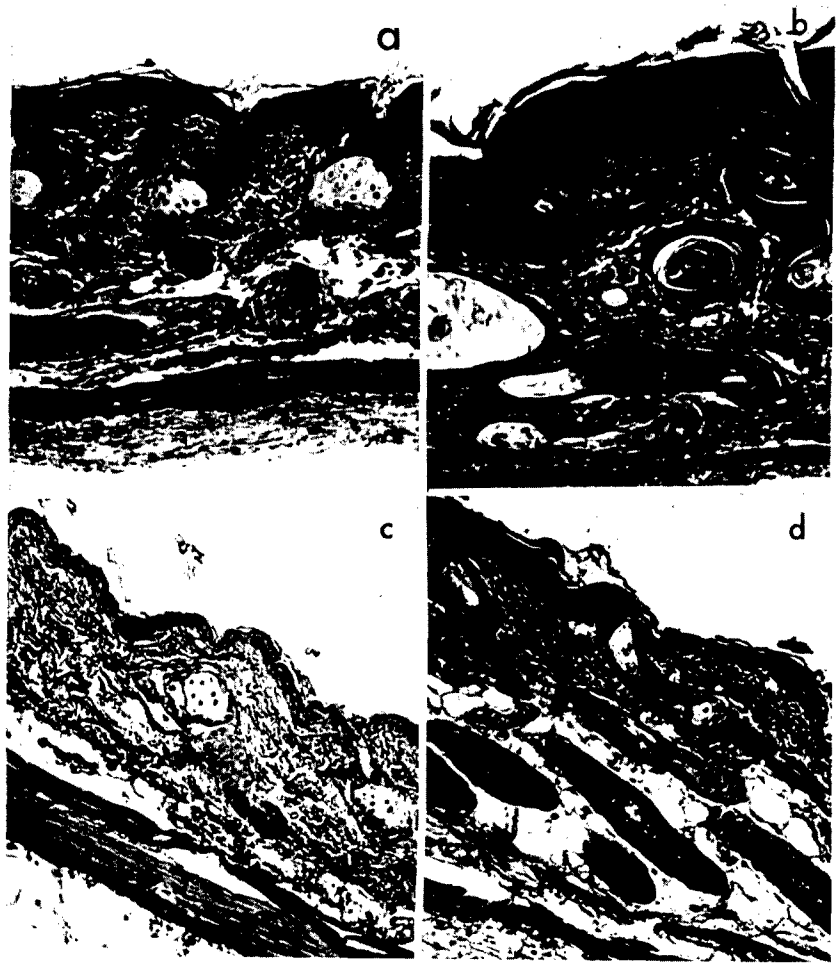

Figure 2. Histology of skin of HRS/J hr/hr and hr/+ mice administered TCDD.

A single application of TCDD (3 nmoles) or acetone was administered to six-week-old female HRS/J mice. Fourteen days later skin samples were taken and processed for histology. a) HRS/J hr/hr, acetone; b) HRS/J hr/hr, TCDD; c) HRS/J hr/+, acetone; d) HRS/J hr/+, TCDD. Note that in hr/hr mouse skin epidermal hyperplasia, sebaceous cell metaplasia and hyperkeratosis is evident, while these skin lesions are absent in hr/+ mice.

TCDD and other receptor agonists induce AHH activity in XB cells plated in the presence or absence of the irradiated 3T3 feeder layer (Table 1). In contrast, the keratinization response to TCDD requires that the XB cells be cultured with the feeder cells or media in which 3T3 cells had been previously cultured. The AHH activity measured in XB/3T3 cells cultured in the presence of TCDD and 7,8-benzoflavone, an inhibitor of cytochrome P-450

TABLE 1. EFFECT OF IRRADIATED 3T3 CELLS ON THE BIOLOGICAL RESPONSE OF XB CELLS TO TCDD

Cells	Compounds	AHH Activity[a] (fmol/min/plate)	Keratinization[b]
XB+3T3	DMSO	340	-
	TCDD	4530	+
XB	DMSO	770	-
	TCDD	5250	-

a) XB cells (4×10^5) and irradiated 3T3 cells (8×10^5) or XB cells (10^6) alone were plated in 100 mm dishes. DMSO or TCDD (10^{-9} M) was added on day 3, and 48 hours later the cells were harvested and assayed for AHH activity (Knutson and Poland, 1980a). Each value represents the average from two cultures.

b) XB cells (10^5) were plated with or without irradiated 3T3 cells (3×10^5) in 60 mm dishes. DMSO or TCDD (10^{-9}M) was added with each medium change. Plates were stained with Rhodanile blue after two weeks (Knutson and Poland, 1980b).

TABLE 2. EFFECT OF 7,8-BENZOFLAVONE ON INDUCTION OF AHH ACTIVITY IN XB CELLS

Compound	AHH Activity[a] (fmol/min/plate)
DMSO	100
TCDD (10^{-9}M)	3840
TCDD + 7,8-Benzoflavone (10 μM)	150

a) XB cells (1.25×10^5 per 100 mm plate) were plated with lethally irradiated 3T3 cells (8×10^5 per plate). Compounds were added on day 4, and 48 hours later the cells were harvested and assayed for AHH activity. Each value represents the average from two cultures.

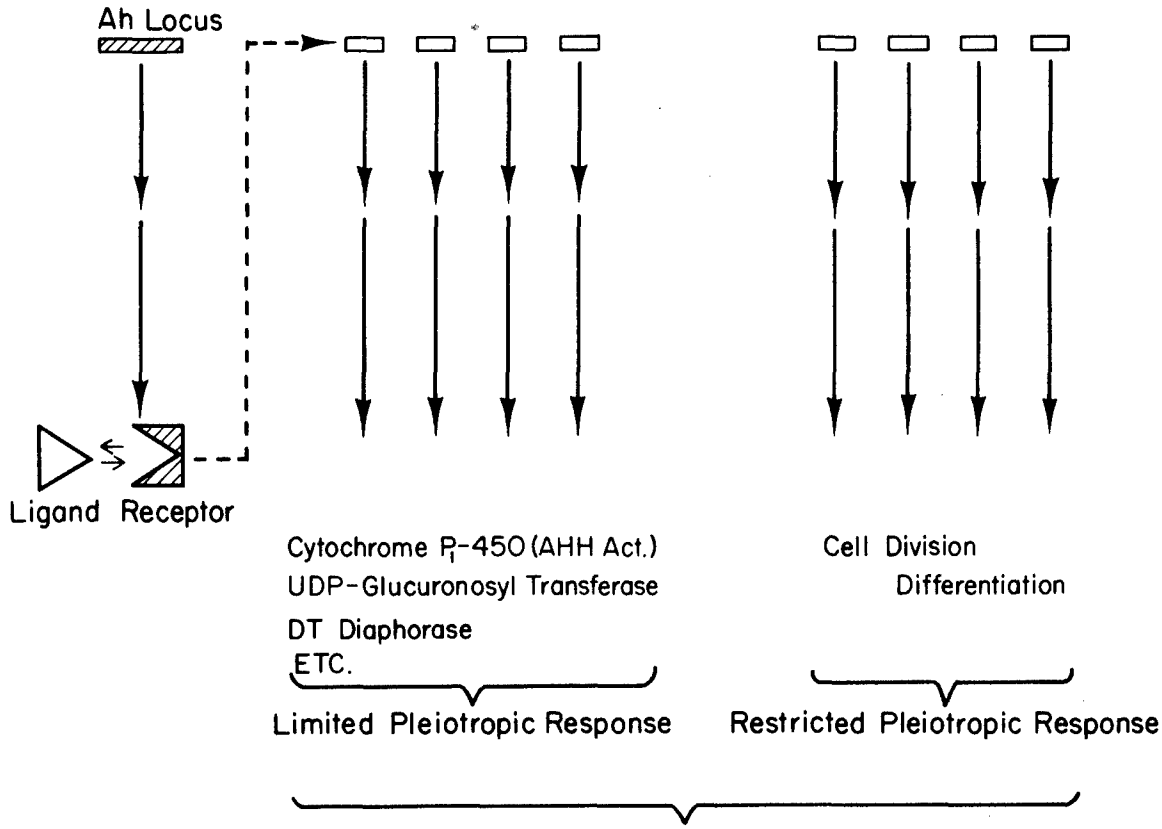

Figure 3. Model for two distinctive pleiotropic responses to the halogenated aromatic hydrocarbons mediated by the cytosol receptor.

The Ah locus determines the cytosol receptor that binds halogenated aromatic hydrocarbons and mediates the ensuing gene expression. In the epidermis of HRS/J (hr/+) mice or in XB cells cultured at high density in fresh medium, there is a limited pleiotropic response, consisting primarily of the induction of enzymes related to drug metabolism (shown on the left of the diagram). In the skin of HRS/J (hr/hr) mice or in XB cells cultured at high density with irradiated 3T3 cells, an additional battery of genes is expressed that is involved in cell division and differentiation (shown on the right of the diagram).

mediated monooxygenase activity, is similar to the activity measured in control cultures (Table 2). Nevertheless, TCDD produces keratinization in XB/3T3 cells incubated with 7,8-benzoflavone. For the halogenated aromatic hydrocarbons tested, the structure activity relationship for induction of AHH activity in XB cells corresponds with that for binding to the cytosol receptor and for producing keratinization.

A GENERAL MODEL FOR TOXICITY

In HRS/J mouse epidermis and mouse teratoma cell line XB, TCDD (and congeners) produces two distinctive pleiotropic responses: a) the induction of enzymes primarily involved in drug metabolism, typified by AHH activity, and b) a response which results in cell division/differentiation. Both responses are mediated by the cytosolic receptor. The induction of AHH activity occurs in the epidermis of both haired (hr/+) and hairless (hr/hr) HRS/J mice or in XB cells cultured with or without a 3T3 feeder layer. The more extensive histologic response occurs in hairless (hr/hr) HRS/J mice or XB cells cultured with 3T3 cells, but is unexpressed, i.e. restricted, in haired (hr/+) HRS/J mice and XB cells cultured alone in fresh medium. These results are shown diagramatically in Figure 3.

Many lesions produced by TCDD and congeners, especially those involving proliferation and/or differentiation of epithelial tissues, occur in a limited number of species. For instance, hyperplastic/metaplastic changes are produced in the skin in humans, monkeys, and rabbits, but not mice, rats, hamsters or guinea pigs; in the urothelium in guinea pigs, monkeys and cows, but not mice and rats; and in the gastrointestinal epithelium in monkeys and hamsters, but not mice, rats or guinea pigs. Yet, many of these tissues which fail to develop histologic changes, e.g. the skin and gastrointestinal epithelium of rats and mice, respond to TCDD with the induction of AHH activity and other coordinately expressed enzymes. Thus, the species specificity of some lesions appears analogous to the specificity of the histologic changes in the epidermis of HRS/J mice.

We suggest that the model developed for the response of HRS/J epidermis to TCDD can be viewed as a general model for the mechanism of toxicity of the halogenated aromatic hydrocarbons. For cells and tissues which possess the receptor, essentially all respond to TCDD and congeners with a limited response consisting primarily of enzymes related to drug metabolism. The histopathologic lesions occur only in those tissues in which an additional battery of genes is expressed. This additional battery of genes regulate cell involution, altered differentiation, or proliferation, and their sustained expression produces the observable toxic lesions. Tissues which have the receptor, but do not display toxicity, may possess these additional genes, but fail to express them in response to TCDD.

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PAPER NO. 17

THE STARVATION-LIKE SYNDROME AND 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN: NEW IDEAS ON THE MODE OF ACTION AT THE WHOLE ANIMAL LEVEL

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INTRODUCTION

TCDD and related compounds cause a starvation-like or wasting syndrome in several animal species. In young animals this response is manifested as a cessation of weight gain whereas in adults it is characterized by weight loss. The dose of TCDD required to elicit the response varies widely between species, as does the lethal dose (McConnell, 1980). However, if a dose of TCDD approaching the LD_{50} for a species is administered, a cessation of weight gain or a loss of weight is consistently observed. This progressive response occurs in laboratory animals (mouse, hamster, rat, guinea pig, rabbit, dog, monkey), farm animals (chicken, cow, horse), and wildlife (mink, guppy, rainbow trout, coho salmon, northern pike). The mechanism by which TCDD causes weight loss is unknown. It is the purpose of this paper to discuss the role of altered food intake, oxygen consumption, and gastrointestinal absorption in the weight loss response. In addition, we will present evidence which suggests that TCDD-induced weight loss in the rat occurs secondary to a reduction in the animal's set-point for regulated body weight.

THE ROLE OF FOOD INTAKE IN TCDD-INDUCED WEIGHT LOSS

Harris and coworkers (1973) reported that sublethal doses of TCDD caused weight loss in rats but did not significantly reduce food intake. Subsequent studies in rats (Allen et al., 1975; Van Logten et al., 1981), guinea pigs and mice (McConnell et al., 1978a), and monkeys (McConnell et al., 1978b) implied that food intake was unaltered until just before death, at which time it was decreased. Chronic feeding studies in mice and rats (Gupta et al., 1981), monkeys (Allen et al., 1977), and cattle (McConnell et al., 1980) also showed that food intake was not altered by TCDD or related compounds even though the animals lost weight. The only chronic exposure study which reported that TCDD lowered the level of food intake in rats was that of Kociba et al. (1976). They discovered that the phenomenon occurred at only the highest chronic dose of

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TCDD used. Together, these results have given rise to the idea that reduced food intake is not the major cause of weight loss in TCDD-treated animals.

Our results are not in agreement with this current dogma. More specifically, we have found that TCDD administered as a single, oral dose of 5, 15, 25 or 50 µg/kg to male rats (300 g) produced a dose dependent decrease in food intake and body weight. The reduction in food intake was detected immediately after treatment and persisted for the duration of the 35 day study. In rats given 5 µg/kg, the greatest decrease in feeding occurred between days 3-6 post treatment. Food consumption then began to recover and eventually stabilized at a level only slightly below that of control rats. In the 15 µg/kg group, the greatest depression occurred between days 6 - 13 after treatment. Food intake increased thereafter and plateaued below that of the 5 µg/kg group. The reduction in feeding was more substantial at the 25 µg/kg dose where it reached its lowest level 8-15 days after TCDD administration and then showed only a slight recovery back to the control level. The most pronounced depression in food intake was seen in rats given 50 µg/kg. By days 14-18 after TCDD treatment, intake in these rats was less than 40% of the control animals and showed no sign of recovery. Lethality was not observed in rats treated with 5 and 15 µg/kg of TCDD. However, in the groups treated with 25 and 50 µg/kg, cumulative lethality at day 35 was 25 percent and 75 percent, respectively. The median time to death in the 25 and 50 µg/kg groups was 28 and 20 days, respectively. Body weight at the time of death was approximately 50 percent of the control group.

To determine whether the persistent depression in feeding was the result of a direct effect of TCDD, or simply due to the reduced body mass of the rats, food intake was expressed relative to the animal's metabolic body size (MBS). MBS is an estimate of the animal's mass of metabolically active tissue, and is defined as body weight in kilograms raised to the 0.75 power. Our rationale for expressing food intake as g food/MBS/day is that resting metabolic rate is directly proportional to MBS (Kleiber, 1975). Since resting metabolic rate constitutes a large portion of an animal's basic energy needs, it follows that the amount of food required per day to meet these needs and therefore maintain body weight should also be proportional to MBS. When food intake was expressed relative to MBS there was a dose-dependent depression for up to 20 days after TCDD treatment. However, from 20-35 days food intake/MBS/day was similar in control rats and rats treated with 5 and 15 µg/kg of TCDD. This indicates that the groups treated with non-lethal doses of TCDD were consuming the appropriate amount of food for their reduced body weight. In other words, these rats were regulating food intake as if they were at their proper level of maintained weight. Food intake/MBS/day of rats treated with lethal doses of TCDD, 25 and 50 µg/kg, never returned to a level that was sufficient to maintain their body weight and these animals eventually died.

Our finding that acute, sub-lethal doses of TCDD cause an immediate and significant depression in food intake is contrary to the finding of Harris et al., 1973. The latter investigators showed that sublethal doses of TCDD do not significantly depress food intake in rats. We think this discrepancy might have been caused by differences in the methods used to measure food intake. In the study by Harris and coworkers (1973), food consumption was measured by the disappearance of blocks of food from a suspended stainless steel feeder. There was apparently no attempt made to account for food that was removed from the feeder and not consumed by the rat. In our study, rats were housed individually in suspended cages. Each animal was provided with 40 g of ground Purina Rat Chow per day which was placed in a food cup wired to the inside of the cage. Since ground food was used, a plastic pan was placed beneath each cup to collect food that was spilled by the rats while feeding. We observed that rats treated with 25 or 50 µg TCDD/kg spilled more food than control rats. The increase in spillage was first detected 6 days after treatment and steadily increased until the TCDD-treated rats were spilling 2-3 times more food than control rats. This was not an insignificant effect inasmuch as the amount of spilled food 15 days after treatment for the 25 and 50 µg/kg TCDD groups was approximately 12 and 18 g/day as compared to 5 g/day for the control group. If these differences in spillage were not accounted for, food intake in TCDD-treated rats would have been overestimated.

THE ROLE OF WHOLE ANIMAL OXYGEN CONSUMPTION IN TCDD-INDUCED WEIGHT LOSS

There is no evidence to support the notion that TCDD-treated rats are hyperthyroid and that the weight loss is secondary to an increase in whole animal oxygen consumption. In fact, the opposite appears to be true. Bastomsky (1977) reported that nine days after male rats were treated with 25 µg/kg of TCDD they exhibited reduced serum thyroxine concentrations, elevated serum thyrotropin concentrations, enhanced uptake of radioactive iodide by the thyroid gland, and increased thyroid gland weight. All of these effects imply that TCDD-treated rats might be hypothyroid relative to control rats. In light of these findings, we sought to determine if oxygen consumption measured in the whole animal was altered at designated times after TCDD treatment. Spontaneous motor activity and oxygen consumption were quantitated in adult male rats treated with a single dose of vehicle (control) or 15 or 50 µg/kg of TCDD. On days 2, 7, 12, 17, 22 and 27 after treatment, motor activity and oxygen consumption were monitored continuously in each animal for 24 hours. The methods used were described in detail by Seefeld and Peterson (1982).

Oxygen consumption in the whole animal was expressed in two ways: as total oxygen consumption which estimates oxygen consumption during periods of motor activity as well as inactivity, and as resting oxygen consumption which is measured only during periods of

prolonged motor inactivity. The main findings were that TCDD caused a dose-related decrease in motor activity and total and resting oxygen consumption. This was seen for oxygen consumption in both TCDD treatment groups when the results were expressed as ml O_2/min/rat. To determine if the depression in total and resting oxygen consumption was secondary to the reduced body weight of the TCDD-treated animals, it was necessary to express oxygen consumption relative to each rat's metabolic body size (ml O_2/MBS/min). When this was done, we found that rats treated with 15 µg/kg were consuming the same amount of oxygen relative to MBS as control rats by day 17 post treatment. In other words, rats treated with 15 µg/kg of TCDD were consuming the appropriate amount of oxygen for their reduced level of body weight. On the other hand, rats treated with the lethal dose of TCDD, 50 µg/kg, consumed less oxygen than the other two groups at all times after treatment. This effect was seen regardless of whether oxygen consumption was expressed as ml O_2/min/rat or as ml O_2/MBS/min. The significance of these findings is that they show that an increase in metabolic rate is not the cause of weight loss.

THE ROLE OF GASTROINTESTINAL ABSORPTION IN TCDD-INDUCED WEIGHT LOSS

Another factor that has been considered as a potential cause of the weight loss in TCDD-treated animals is decreased absorption of nutrients from the gastrointestinal tract. The following findings are typically cited as indirect evidence supporting a malabsorption hypothesis. First, pair-feeding studies showed that rats and guinea pigs treated with lethal doses of TCDD lost weight at a greater rate and to a greater extent than pair-fed control animals (Van Logten, Gupta and Moore, unpublished results cited by McConnell et al., 1978a; Gasiewicz and Neal, 1979; Gasiewicz et al., 1980). Second, weight loss in TCDD-treated rats was not prevented when a diet of pelleted feed was supplemented with a balanced liquid diet administered 3 times per day by gavage (Courtney et al., 1978). Third, when gastrointestinal absorption is bypassed by feeding rats intravenously with essential nutrients, the weight loss response normally elicited by TCDD is prevented (Gasiewicz et al., 1980).

The effect of TCDD pretreatment on absorption of nutrients from the gastrointestinal tract has been directly assessed in vitro using the everted intestinal sac technique. Ball and Chhabra (1981) reported that glucose and leucine absorption was depressed in jejunal segments taken from rats treated with 100 µg TCDD/kg. A depression of intestinal glucose absorption was also observed in mice treated with 75-300 µg TCDD/kg (Madge, 1977). Additionally, it has been suggested that the absorption and assimilation of dietary liquid by the intestine is depressed in rats given 80 µg TCDD/kg (McConnell and Shoaf, 1981; Shoaf and Schiller, 1981).

The main problem in accepting malabsorption as the principal cause of weight loss in TCDD-treated animals is that most of the studies used only lethal doses to show a depressant effect on absorption. Weight loss also occurs after sublethal doses, but there is no evidence that gastrointestinal absorption is depressed sufficiently by these lower doses to account for the weight loss. Furthermore, a study by Neal et al. (1979) does not support a malabsorption mechanism of weight loss. These investigators measured $^{14}CO_2$ production in pair-fed control and TCDD-treated guinea pigs that were given ^{14}C-glucose, ^{14}C-alanine or ^{14}C-oleate orally one week after treatment with an LD_{50} dose of TCDD. At this time the TCDD-treated guinea pigs had lost approximately 25% of their original body weight, yet their ability to metabolize glucose, alanine and oleate to CO_2 was similar to that of pair-fed control guinea pigs. It was concluded that there was no generalized depression in the absorption of these compounds from the intestine of TCDD-treated guinea pigs.

The results of a study conducted in our laboratory suggest that absorption of calorie-containing nutrients from the gastrointestinal tract of rats is not depressed by TCDD. Rats were treated with 0, 15 or 50 µg/kg of TCDD. The total number of calories consumed in food and eliminated in feces were determined on days 3, 7, 14, 21 and 28 post treatment by direct calorimetry. As expected from the depression in food intake, there was a corresponding depression in caloric intake dose related and persisted for the 28 day duration of the study. The new finding was that the percent of the caloric intake excreted in feces was similar for control and TCDD-treated rats, about 20%. Thus, TCDD-treated rats did not excrete more calories in their feces than control rats, as would have been expected had a nonspecific malabsorption syndrome occurred.

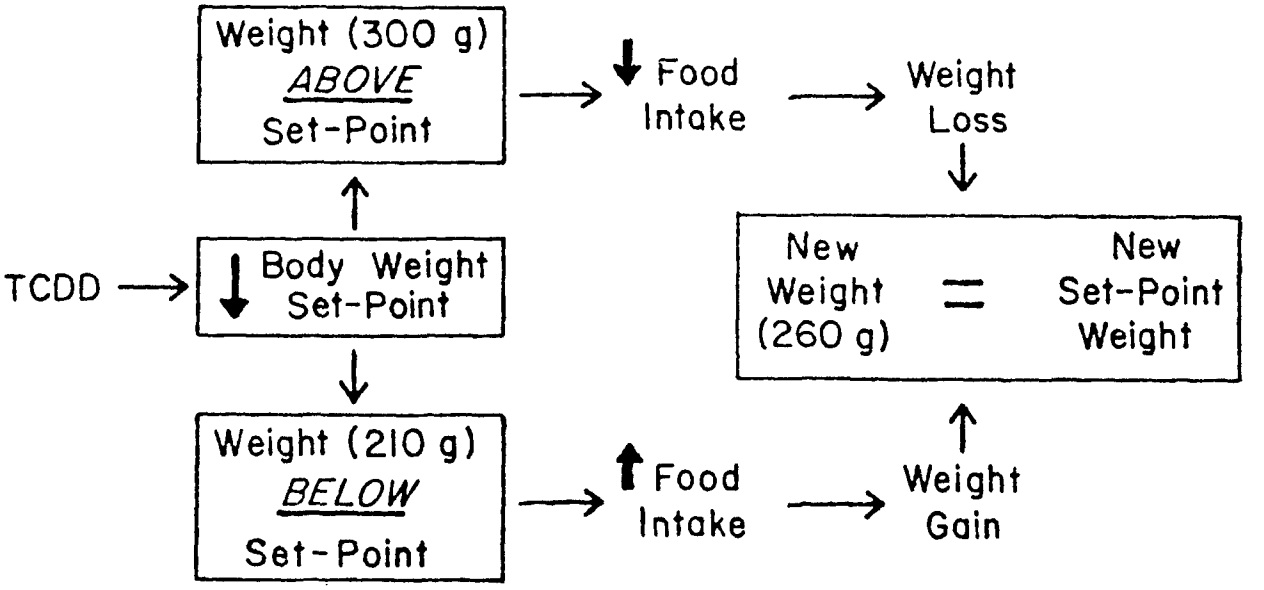
It must be emphasized that the preceding calorie balance study does not provide information on the absorption of specific nutrients. In this respect it is entirely possible that TCDD treatment depresses the absorption of certain nutrients by the intestine without altering the amount of calories excreted by the animal in feces. Therefore, it was essential that the calorie balance study be followed by pair-feeding studies. If TCDD treatment caused weight loss by impairing the absorption of certain key nutrients from the gastrointestinal tract, then the weight loss of TCDD-treated rats should be greater than that of pair-fed control rats. We have conducted such pairfeeding studies in adult rats treated with 15, 25 and 50 µg TCDD/kg and in weanling rats treated with 25 µg TCDD/kg. All rats were fed a ground chow diet and care was taken to correct food intake of the TCDD-treated rats for spilled food. In all cases, body weight of the pair-fed control group was only slightly greater than that of the TCDD group. This shows that reduced food intake, not malabsorption, is the main cause of weight loss. Again, differences in the pair-feeding results in our study as opposed to earlier studies may be due to differences in the way food intake was measured.

A REDUCTION IN THE REGULATION LEVEL OR SET-POINT FOR BODY WEIGHT

Having established that the TCDD-induced depressions in food intake and body weight have similar dose and time dependencies, we now propose a mechanism to explain the close association of the two responses. Our hypothesis is that TCDD causes a dose-dependent decrease in the regulation level or setpoint for body weight. This mechanism is based on the concept that body weight in rats is regulated around a set-point value. Existing weight is constantly being compared to the set-point weight and if a difference exists, food intake is adjusted to raise or lower body weight until it matches the set-point weight. We suggest that by lowering the body weight set-point, TCDD creates a mismatch between the set point and existing weight. This leads to decreased food intake and weight loss until the mismatch is corrected. Once corrected, TCDD-treated rats are postulated to maintain weight at the reduced level determined by their lower body weight set-point. Thus, while at the lower weight level, TCDD-treated rats appear to regulate their body weight in the same way that control rats of the same age and sex regulate their body weight. The only difference is that the same regulatory adjustments take place around a lower level of body weight in the TCDD-treated animal.

POST TREATMENT ADJUSTMENTS IN BODY WEIGHT BY RATS TREATED WITH TCDD WHILE AT A NORMAL AND REDUCED WEIGHT LEVEL

To test the hypothesis that TCDD decreases the set-point for regulated body weight, the study diagrammed below was conducted. Male rats (250 g) were divided into two groups. One group was fed ground food ad lib for 7 days and gained weight to 300 g. The food intake of these rats was approximately 25 g/day. The other group was placed on a restricted feeding schedule (10 g food/day) to reduce body weight. After 7 days, the mean weight of these rats was 210 g or 70% of the weight of the ad lib fed rats. At this time both groups were treated with a single, oral dose of TCDD (25 µg/kg) and allowed thereafter to feed ad lib. The rats whose body weight was 300 g exhibited immediate hypophagia and weight loss after TCDD until their weight leveled off at 260 g. The animals whose weight had been reduced to 210 g displayed a different post treatment pattern. Rather than becoming hypophagic after TCDD, these animals displayed hyperphagia relative to the 300 g group and gained 50 g during the 12 day period immediately following TCDD treatment. Approximately 12-15 days post treatment, both groups converged at the same reduced level of weight (260 g) and consumed comparable amounts of food thereafter. Thus, the post treatment feeding behavior of the TCDD-treated rats was appropriate to the achievement of the new level of body weight determined by the dose of TCDD. If treated at a weight that was normal for the age and sex of the animal, the rats become hypophagic and lost weight. On the other hand, if treated while below the weight level determined by the dose



of TCDD, the animals became hyperphagic and gained weight. If TCDD was directly affecting the rat's ability or willingness to feed, then food intake in the two groups should have been similar, not different as was observed. Thus, TCDD does not appear to directly inhibit a system controlling food intake.

Rather, we suggest that TCDD regulates a system controlling body weight. In this context, the well coordinated adjustments in feeding behavior, hypophagia and relative hyperphagia, are viewed as secondary responses that are necessary in order for the rat to achieve the reduced level of body weight determined by TCDD. The primary response which sets the entire process in motion is considered to be the reduction in body weight set-point. We propose that the reduction in set-point is a graded response that is directly related to the dose of TCDD administered.

PERSISTENCE OF THE LOWER LEVEL OF MAINTAINED WEIGHT IN TCDD-TREATED RATS

The reduced body weight of the TCDD-treated rats is not sufficient evidence in itself of an altered regulation level or set-point for body weight. In addition, it must be shown that the lower weight level is (1) maintained chronically, and (2) precisely defended when the animal's body weight is displaced from the lower weight level. For these reasons, we have conducted a series of experiments to determine whether the reduced body weight of rats treated with TCDD is persistent over time and defended when challenged with various dietary and body weight manipulations.

To determine the persistence of TCDD's depressant effect on body weight, rats were treated with a single, oral dose of 15 μg/kg of TCDD or vehicle. Following this dose of TCDD, rats displayed hypophagia and weight loss for the first three weeks after treatment. Body weight then stabilized, and by the middle of the fourth week a pattern of weight gain reappeared. This enabled the TCDD group to maintain a level of weight for the remainder of the thirteen week post treatment period that was approximately 85% of that maintained by the age and sex matched control group. As the TCDD-treated rats approached the 85% level of weight maintenance, their food intake returned to a level that was approximately 2-3 g/day less than that of the control group. Food intake then remained at this slightly lower level for the duration of the post treatment period. Thus, the requirement that the reduced level of body weight be maintained for an extended period of time in order to demonstrate a TCDD-induced reduction in the body weight set-point was satisfied.

DEFENSE OF THE LOWER LEVEL OF MAINTAINED WEIGHT IN TCDD-TREATED RATS AGAINST DIETARY MANIPULATIONS

To determine if the TCDD-treated rats defend their reduced body weight with the same precision that control rats display in defending a normal weight level, several challenge experiments were conducted. The rats used in these experiments were subjected to various dietary and body weight manipulations beginning three weeks after treatment with a single dose of 15 μg/kg of TCDD or vehicle (control). At this time TCDD-treated rats were maintaining a level of weight that was approximately 85% of that maintained by control rats.

In the first experiment, TCDD-treated and control rats that had been maintained on a diet of ground food and water for three weeks after treatment were switched to a highly palatable eggnog diet. The objective of this experiment was to observe the effect of the eggnog diet on body weight and to determine the adjustments made by each group as the caloric density of the diet was altered by progressively diluting it with water. The eggnog was diluted to 90% from weeks three to seven after TCDD, to 67% on week eight and to 45% on week nine. From weeks ten to thirteen after TCDD, the two groups were returned to the less palatable diet of ground food and water. When the eggnog diet was first introduced, body weight of both the TCDD and control groups increased markedly and eventually stabilized at higher weight levels. As the caloric density of the eggnog was progressively diluted, both groups maintained their body weights at the higher levels even though daily intake had to be increased substantially. When returned to the less palatable diet of ground chow, the weights of both groups declined in parallel and plateaued at lower maintenance levels. Throughout the six-week period of dietary manipulations, the mean body weight of the TCDD group was 85 ± 1% of that of the control group.

Similar results were obtained when a diet of ground chow was made unpalatable by the addition of quinine sulfate (0.3%). Both groups of rats exhibited a reduction in body weight after being fed this diet for four weeks. Percent weight loss in the TCDD group was 11.6% and in the control group 13.5%. Hence, body weight in both groups was reduced in a similar manner by the unpalatable diet.

DEFENSE OF THE LOWER LEVEL OF MAINTAINED WEIGHT IN TCDD-TREATED RATS AGAINST BODY WEIGHT MANIPULATIONS

The ability of the TCDD-treated rat to defend its lower weight maintenance level when the animal's body weight was displaced from that level is another criterion for a reduction in body weight set-point. In the subsequent studies, body weight manipulations were initiated three weeks after treatment, with 15 μg/kg of TCDD or vehicle (control). At this time, body weight of the TCDD group was 85% that of the control group.

In the first study, we evaluated the ability of TCDD-treated rats to defend their lower level of weight against a food restriction challenge. For this experiment we subdivided both the TCDD and control groups such that half of the rats were allowed to feed ad lib and the other half were food restricted for ten days. By the end of the ten day period mean body weight of the food restricted TCDD and control groups had been reduced to about 80% of the level of weight maintained by their ad lib fed counterparts. The food restricted animals were then returned to an ad lib feeding schedule and their body weight and food intake were closely monitored. The main finding was that the two food restricted groups exhibited similar patterns of weight recovery and returned to the levels of weight maintained by their ad lib fed counterparts. In the TCDD group the period of weight recovery was approximately 23 days while in the control group it was 19 days. In addition, weight recovery in both groups was associated with a similar change in feeding behavior. More specifically, when returned to the ad lib feeding schedule, both food restricted groups displayed hyperphagia relative to their ad lib fed counterparts. As body weight of the previously food restricted groups converged with their respective ad lib fed groups, so did their food intakes. Thus, both TCDD-treated and control rats rapidly returned to their appropriate weight level following weight loss and did so by making similar adjustments in food intake.

In another weight manipulation experiment, TCDD and control groups were again subdivided three weeks post treatment. Half of the rats in each treatment group were fed ground food ad lib while the other half were fed a highly palatable, calorie-rich, eggnog diet ad lib. The TCDD and control groups fed eggnog gained weight at a similar rate over the four week period that the highly palatable diet was available. By the end of the fourth week, body weight of the TCDD and control groups fed eggnog had increased 14% and 12% above the level of weight maintained by the TCDD and control groups

fed ground food ad lib, respectively. When returned to ground food, both eggnog fed groups immediately lost weight and returned to the weight levels maintained by their respective counterparts. Hence, both TCDD-treated and control rats increased their body weights above their levels of maintenance in a comparable manner when fed an eggnog diet. Furthermore, when switched back to a less palatable, ground chow diet, each group lost weight until it returned to its appropriate weight maintenance levels.

CARCASS COMPOSITION OF TCDD-TREATED AND CONTROL RATS

To obtain greater insight into the mechanism of TCDD-induced weight loss the carcasses of control and TCDD-treated rats were analyzed for fat, protein, water and ash. The carcass composition analyses were done seven weeks after treating male rats with a single, oral dose of TCDD (15 μg/kg) or vehicle (control). During the seven week period both groups were fed a diet of ground food and water ad lib. When the rats were sacrificed the body weight of the TCDD group was 85% of that of the control group. Prior to analyzing the carcasses, the contents of the gastrointestinal tract were removed. The lower carcass weight of the TCDD groups was found to be due to a reduction in fat, protein and water. The amount of ash was similar in the two groups. Another way to compare carcass composition is to express the weight of each constituent as a percentage of carcass weight. In so doing we found that the percentage of fat, protein, water and ash was similar in the two groups seven weeks after treatment. This latter finding suggests that TCDD-treated rats regulate body fat, protein and water in the same fashion as control rats when both groups are at their respective levels of weight maintenance.

To determine the effect of feeding a high calorie diet on carcass composition, we included two other groups as part of the previous study. These rats were treated with 15 μg/kg of TCDD or vehicle (control). For the first three weeks post treatment both groups were fed ground food. While on this diet the body weight of the TCDD group was reduced to a level that was 85% of that of the control group. Both control and TCDD groups were then fed a 90% eggnog diet for four weeks. While on the eggnog diet, a parallel increase in weight gain was observed in the two groups. At the end of this time the animals were sacrificed. It was found in both groups that the excess weight gained while on the eggnog diet was due to an increase in carcass fat and water. Thus, the eggnog diet was equally lipogenic in control and TCDD-treated rats.

To further evaluate the effect of TCDD on body weight regulation, we compared carcass composition of TCDD-treated rats whose body weight had been returned to the level maintained by control rats fed ground food ad lib. This was accomplished by feeding the TCDD group a 90% eggnog diet. We found that carcass composition was different in the two groups even though body weight was the same.

The difference in carcass composition was that the TCDD-treated animals fed eggnog had more fat and less protein and water in their carcasses than did control rats. Thus, returning TCDD-treated rats to a level of body weight that was appropriate for their age and sex did not result in an appropriate carcass composition.

THE ROLE OF STUNTING IN TCDD-INDUCED WEIGHT LOSS

Our previous results have shown that the lower weight level of TCDD-treated rats is maintained chronically and is defended when the animal's body weight is displaced from this lower level. These results support the hypothesis that TCDD decreases the regulation level of set-point for body weight in the rat. However, an alternative explanation is that a reduction in food intake during the first few weeks after TCDD treatment stunts the animals such that catch-up growth is impossible. To test this possibility, we pair-fed control rats to TCDD-treated rats (15 μg/kg) for seven weeks. At the end of the seventh week, body weights of the TCDD and pair-fed control groups were, respectively, 85% and 87% of that of an ad lib fed control group. The pair-fed group was then permitted to feed ad lib for six weeks. During this time the pair-fed group gained weight more rapidly than the TCDD group. This difference in weight gain was clearly evident at the end of the additional six week period when body weights of the TCDD and pair-fed control groups were, respectively, 87% and 98% of that of the ad lib fed group. Thus, we do not think that stunting, secondary to reduced food intake, is responsible for the lower level of body weight of the TCDD-treated rat. If a stunting mechanism were involved, then the pair-fed control group should not have been able to restore its body weight to the level of the ad lib control group.

CONCLUSION

In closing, we wish to impress upon the reader that the depressant effect of TCDD on the body weight set-point is probably reversible. As TCDD is eliminated from the animal, its depressant effect on the set-point will likely subside. Therefore, it is feasible that rats treated with a single, sublethal dose of TCDD will eventually recover to a level of weight maintenance appropriate to their age and sex. Furthermore, the site of action and mechanism by which TCDD causes the down regulation in the set-point are unknown. Only through further research will this information become available.

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PAPER NO. 18

RECEPTORS FOR 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN: THEIR INTER- AND INTRA-SPECIES DISTRIBUTION AND RELATIONSHIP TO THE TOXICITY OF THIS COMPOUND

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INTRODUCTION

Since the 1950's, the chlorinated dibenzo-p-dioxins and related compounds, including the halogenated biphenyls, dibenzofurans, as well as azo- and azoxy-benzenes, have received considerable attention as toxic environmental contaminants. This has been due primarily to numerous accidental poisonings of human and animal populations (Kimbrough, 1974; Poland and Kende, 1976; Reggiani, 1980) as well as their extreme toxic potency observed in a variety of laboratory animals (Schwetz et al., 1973). Recently, studies by Pitot and co-workers (1980) suggest 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) to be an extremely potent tumor promoting agent. These and related studies have motivated additional interest in this class of compounds as molecular and biochemical probes.

In spite of much research, the exact biochemical mechanism(s) of this class of compound remain(s) as yet unknown. However, evidence accumulated within the past six years suggests the toxicity of these compounds is mediated through specific binding to a cytosolic protein or receptor molecule.

The present discussion will specifically focus on TCDD, the most potent of this class, and briefly put in perspective the evidence for this hypothesis. In addition, recent data on the inter- and intra-species distribution and properties of these receptors will be discussed in relation to the toxicity observed. It is our belief that whatever is the mechanism of toxicity of these compounds, an understanding of the etiology of species differences in susceptibility is most relevant to the determination of their relative toxicity to humans.

EVIDENCE FOR THE ROLE OF A CYTOSOLIC RECEPTOR PROTEIN IN THE TOXICITY OF TCDD

At present, there are two main lines of evidence which suggest that the toxicity of TCDD and related compounds is mediated through binding to a cytosolic receptor protein. The first comes primarily from the laboratory of Dr. Alan Poland and is based on the finding that certain inbred strains of mice have cytosolic binding proteins for TCDD with markedly differing affinities for this compound (Poland et al., 1976). The "non-responsive" strains (typified by DBA/2J mice) appear to have an altered receptor with much lower affinity for TCDD as compared with the "responsive" strains (typified by C57BL/6J mice). Further work suggested an association between receptor binding and the ability of TCDD to induce thymic atrophy, teratogenic effects, and porphyria, as well as lethality in these strains of mice [Table 1] (Poland and Glover, 1980; Jones and Sweeney, 1980; Neal et al., 1982).

TABLE 1. TCDD: SEGREGATION OF TOXICITY WITH RECEPTOR BINDING

	Strain of Mouse		
	C57BL/6J	B6D2F$_1$/J[a]	DBA/2J
LD_{50} (µg/kg)[b]	132	300	620
Cleft Palate[c]			
% Incidence at:			
10 µg/kg	3	0	0
30 µg/kg	54	13	2
Thymic Atrophy[c]			
(ED_{50}, µg/kg)	6.4	32.2	257
Urine Porphyrin[d]			
(µg/mouse/24h, at 25 µg TCDD/kg for 11 weeks)	~3	~3	< 0.5

[a] Offspring of C57BL/6J and DBA/2J mice that are heterozygous at the Ah locus.

[b] Neal et al., 1982.

[c] Approximate dose of TCDD which causes 50% thymic atrophy. Estimated from Poland and Glover, 1980.

[d] Jones and Sweeney, 1980.

The difference in receptor binding among these strains of mice was first discovered as a result of an observed correlative difference in the induction of various enzyme systems [most notably the cytochrome P-450 associated aryl hydrocarbon hydroxylase (AHH) activity] following treatment with TCDD or its congeners (Nebert and Gielen, 1972; Poland et al., 1974). Thus, in these strains of mice, the Ah locus, which is the structural gene for the cytosol receptor (Nebert and Jensen, 1979), appears to control the expression of a number of enzyme systems as well as toxic responses.

The second line of evidence is based on the ability of TCDD and its congeners to bind to the cytosolic receptor and produce a variety of toxic responses in mammalian systems other than the mouse. There is a good correlation between the rank order binding of these compounds to the receptor and their ability to produce lethality in guinea pigs, mice, and chick embryos, thymic atrophy in mice, chloracne in hairless mice, as well as keratinization in XB cells in vitro (McConnell et al., 1978; Bradlow and Casterline, 1979; Poland and Glover, 1980a; Poland and Knutson, 1982; Knutson and Poland, 1980). These data are particularly important in establishing the relationship between receptor binding and toxicity in a variety of mammalian species and cell types. These data also predict that compounds which bind to the cytosolic receptor have the potential to elicit a toxic response. Significant variations in this prediction may be due to other factors including rapid metabolism of the ligand in vivo. This may be the case with some of the polycylic aromatic hydrocarbons, such as β-naphthoflavone and 3-methylcholanthrene (Poland et al., 1976).

The proposed general model (Poland and Glover, 1980b) for these relationships postulates that TCDD or its congeners bind to a cytosolic receptor. The ligand-receptor complex translocates to the nucleus, subsequently causing altered gene expression. The resulting increase or decrease in synthesis of critical macromolecules may eventually lead to toxicity [Figure 1].

The nuclear translocation of the TCDD-receptor complex has been observed and appears to be an essential event in the induction process (Greenlee and Poland, 1979; Okey et al., 1980). However, as we do not yet know the nature of the ultimate biochemical lesion leading to toxicity, the induction of any particular enzyme, including AHH, may be merely a biochemical response to altered gene expression and not an event leading to toxicity and tissue damage.

This simple model, in itself and without some qualifications, does not appear to provide a unifying hypothesis for all the biological events observed following TCDD treatment. In particular, there exists large variability in species susceptibility to the lethal effects of TCDD as well as the signs of toxicity observed [Table 2].

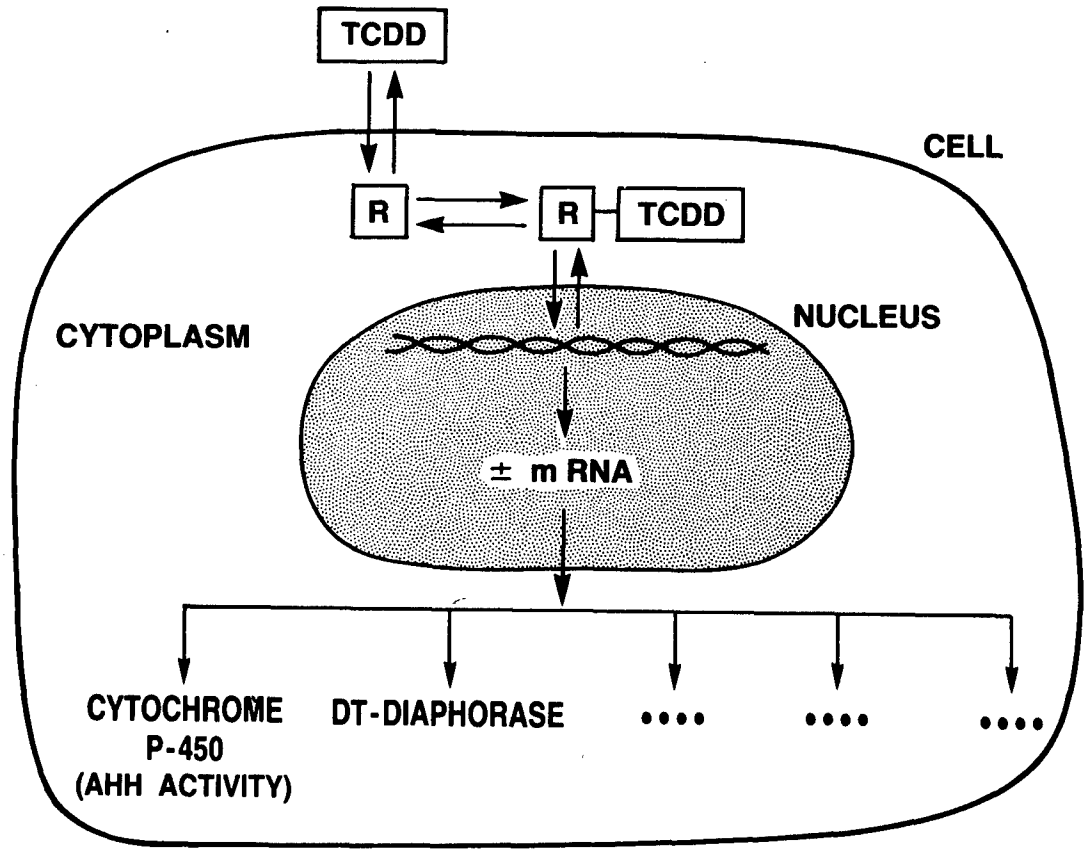

Figure 1. Schematic representation of the proposed model for the interaction of TCDD with its receptor and the resulting coordinate induction of a variety of enzyme systems.

For example, the Golden Syrian hamster appears to be approximately 6000 times less sensitive to the lethal effects of TCDD than the guinea pig. However, despite the sensitivity of the guinea pig, this species does not appear to succumb to demonstrable TCDD-induced morphologic hepatic damage (McConnell et al., 1978) as observed in rats, mice, and rabbits (Gupta et al., 1973; Vos et al., 1974; Vos and Beems, 1971). Notably, the only consistent features of toxicity yet observed are thymic atrophy and loss in body weight. Assuming a role of the receptor in mediating these events, possible variations in relative concentrations, affinities, and/or other properties of the receptor in different tissues and species may account for these observations. Altered properties of the receptor appear to explain the difference in sensitivity of various strains of mice to TCDD. Alternatively, different species and tissues may express a dissimilar battery of genes in response to TCDD exposure. In addition, species or tissue specific biochemical differences may modulate the effects of altered gene expression.

TABLE 2. TCDD: SUMMARY OF TOXIC EFFECTS IN VARIOUS SPECIES[a]

	Guinea Pig	Rat	Monkey	Rabbit	Mouse	Hamster	Man
LD_{50} (μg/kg)	0.6-1.0	25-60	~70	200	100-600	5500	N.D.[b]
Liver Damage	-	++	+	++	+	±	±
Edema	-	-	+	N.D.	+	±	N.D.
Weight Loss	+	+	+	+	+	+	N.D.
Hyperplasia and/ or Metaplasia:							
Intestine	-	-	++	N.D.	-	+	N.D.
Urinary Tract	++	-	++	N.D.	-	N.D.	N.D.
Bile Duct/ Bladder	N.D.	N.D.	++	N.D.	++	N.D.	N.D.
Skin/ (Chloracne)	-	-	++	++	±	-	++
Hypoplasia, Atrophy:							
Thymus	+	+	+	+	+	+	N.D.
Bone Marrow	+	N.D.	+	N.D.	±	N.D.	N.D.
Testes	+	+	+	N.D.	+	N.D.	N.D.

[a] From Poland and Knutson, 1982; McConnell, 1980

[b] N.D. = Not Determined

COMPARISON OF THE BINDING PROPERTIES OF HEPATIC CYTOSOLIC RECEPTORS FOR TCDD IN VARIOUS MAMMALIAN SPECIES

In consideration of the proposed model for the role of the receptor molecule in the toxicity of TCDD, and the wide species differences in susceptibility, we felt it necessary to compare the binding properties of these receptors from various mammalian species. In addition, previous correlations of structure-activity relationships for toxicity in various species and binding to the receptor have used binding data generated only with hepatic cytosol from C57BL/6J mice (Poland et al., 1976).

Utilizing a hydroxylapatite assay system (Gasiewicz and Neal, 1982), we compared the number of receptors as well as their respective binding affinities for TCDD in the livers from male guinea pigs (Hartley), rats (Sprague-Dawley), hamsters (Golden Syrian), a non-human primate (Macaca fascicularis), as well as C57BL/6J, DBA/2J and $B6D2F_1/J$ mice. These results are shown in Table 3. Hepatic receptor concentrations ranged from 23 to 74 fmol/mg of cytosolic protein. With the exception of the three strains

TABLE 3. CONCENTRATION [n] AND APPARENT[a] DISSOCIATION CONSTANTS (K_D) OF THE HEPATIC CYTOSOLIC RECEPTOR FOR TCDD IN VARIOUS SPECIES

Species	n(fmol/mg protein)	K_D(nm)
Guinea Pig	59 ± 11	0.06 ± 0.01
Rat	61 ± 23	0.12 ± 0.03
Monkey	42 ± 8	0.26 ± 0.04
Mouse:		
C57BL/6J	74 ± 10	0.29 ± 0.01
B6D2F$_1$/J	23 ± 2	0.42 ± 0.03
DBA/2J	*	*
Hamster	67 ± 22	0.33 ± 0.07

[a] As determined by hydroxylapatite assay

* Unable to be determined

of mice, there appeared to be no relation between measured hapatic receptor and acute LD_{50} values or susceptibility to hepatotoxic effects of TCDD in these species. Similarly to what has been observed by other investigators (Poland et al., 1976; Okey et al., 1979), little specific binding was observed using hepatic cytosol from DBA/2J mice. In this case, the specific binding was 20% to 40% of the non-specific binding and was considered too low to quantitate accurately. Hepatic receptors from all species demonstrated very high affinities for TCDD, ranging from approximately 0.1 to 0.4 nM. Although some differences may exist in these values, they are considered too small to account for the large differences in toxicity observed.

The relative affinity of other compounds for the hepatic cytosolic binding protein in each species was measured by their capacity to compete with the specific binding of ^{3}H-TCDD. These results are shown in Table 4. With few exceptions, relative affinities of these compounds for the receptor in different species were markedly similar.

To further examine the characteristics of TCDD binding proteins in these species, we determined their relative molecular weights by gel-exclusion high performance liquid chromatography (HPLC).[1] Figure 2 shows the profile of total and non-specific

[1] Gasiewicz, T. A. and G. Rucci, submitted for publication.

TABLE 4. HEPATIC CYTOSOLIC BINDING AFFINITY OF DIBENZO-p-DIOXIN CONGENERS AND OTHER COMPOUNDS RELATIVE TO 2,3,7,8-TCDD IN VARIOUS SPECIES

	Guinea Pig	C57BL/6J S.D. Rat	B6D2F$_1$/J Mouse	Mouse	Hamster	Monkey
2,3,7,8-TCDD	100[a]	100[a]	100[a]	100[a]	100[a]	100[a]
TCDF	46	49	39	42	35	54
1,2,3,4,7,8-HCDD	6.6	1.6	0.82	2.5	0.88	2.3
1,2,4-TCDD	0.31	0.066	0.076	0.15	0.067	0.44
DBD	0.0035	0.0018	0.0012	0.0014	0.00048	0.003
BNF	2.2	2.6	5.9	5.9	1.7	4.7
3MC	3.7	4.2	3.8	3.2	4.2	5.4
BP	1.4	0.86	0.44	0.43	0.46	2.3
PB	----	----	----	----	----	----

Abbreviations are:
2,3,7,8-TCDD: 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)
TCDF: 2,3,7,8-Tetrachlorodibenzofuran
1,2,3,4,7,8-HCDD: 1,2,3,4,7,8-Hexachlorodibenzo-p-dioxin
1,2,4-TCDD: 1,2,4-Trichlorodibenzo-p-dioxin
DBD: Dibenzo-p-dioxin
BNF: β-naphthoflavone
3MC: 3-methyl-cholanthrene
BP: Benzo-[a]-pyrene
PB: Phenobarbital

[a]TCDD is assigned a relative binding affinity of 100.

binding of ^{3}H-TCDD to rat hepatic cytosol following HPLC. In this species, specific binding eluted at fractions 35-38 corresponding to a molecular weight range of 220,000 - 280,000 daltons. To further demonstrate the validity of this method, we examined the profiles of ^{3}H-TCDD specific binding to cytosolic fractions from responsive and non-responsive strains of mice [Figure 3]. In this and all other cases, the specific binding closely matched that as determined using a hydroxylapatite system. Table 5 summarizes the results of analysis of hepatic cytosol from different species. Although small differences may exist, qualitatively the molecular weights of the specific binding proteins for TCDD from each of these species appear to be quite similar.

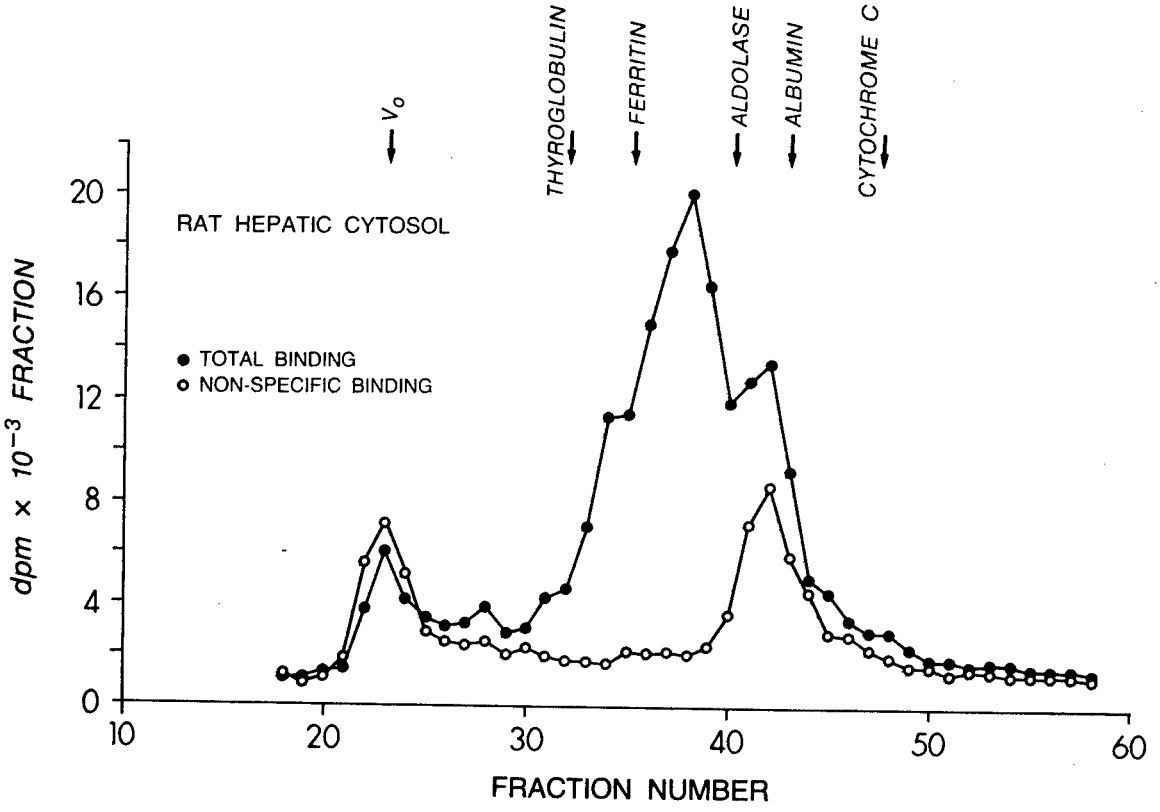

Figure 2. Analysis of TCDD-receptor from rat hepatic cytosol by HPLC. Cytosol (2.85 mg protein/ml) was incubated with ^{3}H-TCDD (1 nM) in the presence [o] (non-specific binding) or absence [●] (total binding) of a 200-fold excess of 2,3,7,8-tetrachlorodibenzofuran. 500 µl cytosol was applied to a TSK 4000 SW HPLC column and eluted with 200 mM potassium phosphate (pH 7.4) buffer at a flow rate of 0.7 ml/min (0.4 min/ fraction). [Specific binding = Total - Non-specific].

These results suggest that despite large differences in toxicity, all species examined possess hepatic cytosolic binding proteins for TCDD with markedly similar properties. In consideration of the structure-activity relationships which exist for toxicity, these results may not be at all surprising. However, these data are important in documenting the presence and marked similarity of these receptors in various mammalian species. In addition, the correlative differences between certain strains of mice in terms of altered specific binding of TCDD and sensitivity to this compound may be a unique situation, not necessarily applicable to other species. Furthermore, and as specifically applied to the liver, these data also favor the postulate that differential hepatic sensitivity to TCDD may in part be due to a variance in the battery of enzymes expressed or repressed following TCDD

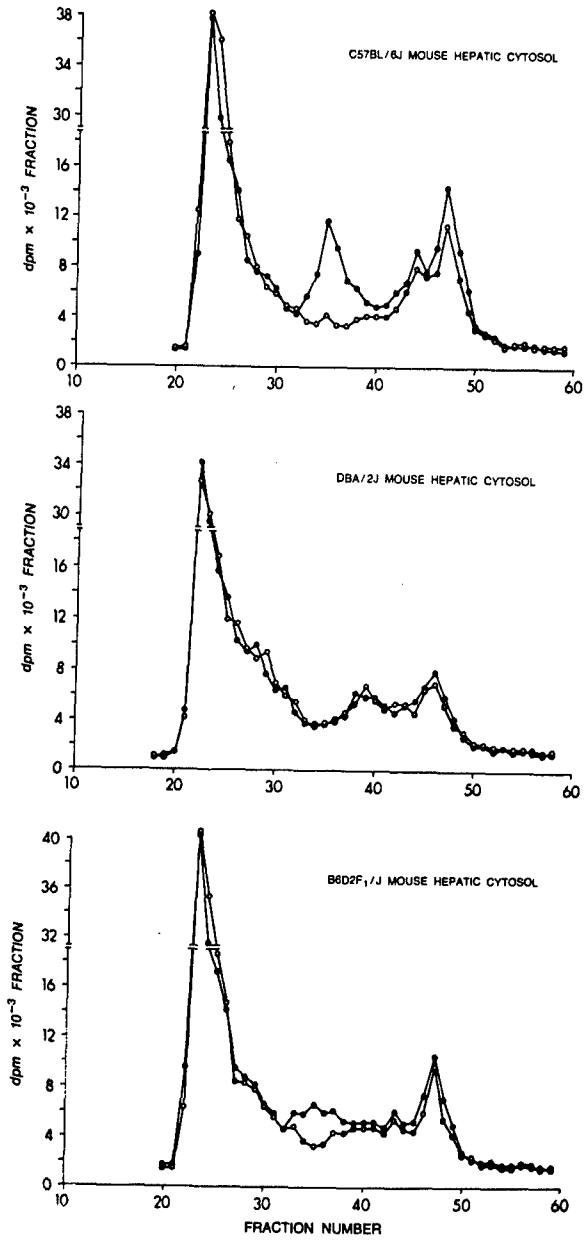

Figure 3. Profiles of total (●) and non-specific (o) binding of ^{3}H-TCDD to cytosolic proteins from C57BL/6J (D), B6D2F$_1$/J (E), and DBA/2J (F) mouse liver. HPLC analysis of the cytosol from each tissue was performed as described in Figure 2.

exposure. It is known that following TCDD administration, AHH activity is induced in hepatic tissue from mice and rats (Poland et al., 1974; Poland and Glover, 1974). Recent evidence[2] suggests that this may also be the case in the hamster. In the guinea pig, although AHH and DT-diaphorase activities are not increased following TCDD treatment, 4-biphenyl hydroxylase activity is increased to a marginal degree (Hook et al., 1975). Recent data also

[2] Olson, J. R. and R. A. Neal, unpublished observations.

TABLE 5. SPECIES COMPARISON OF MOLECULAR WEIGHTS OF SPECIFIC BINDING PEAKS FOR TCDD IN HEPATIC CYTOSOL

Species	Average Percent Recovery Recovery of Molecular Weight Range of Specific Binding Peak	Specific Binding as Determined by HPLC as Compared to Hydroxylapatite Assay
Guinea Pig (Hartley)	220,000 - 280,000	78.0
Rat (Sprague-Dawley)	220,000 - 280,000	94.7
Monkey (Macaca Fascicularis)	280,000 - 300,000	77.9
Mouse (C57BL/6J)	300,000 - 330,000	92.7
Hamster (Golden Syrian)	220,000 - 280,000	84.6

suggest that hepatic enzymes involved in lipid metabolism may be altered in the guinea pig (Swift et al., 1981).[3] Thus, although altered gene expression appears to take place in the liver from all these species, it is only in the livers from mice and rats that those specific systems are expressed (or repressed) which result in hepatotoxicity.

RECEPTORS IN THE THYMUS FROM DIFFERENT SPECIES

One must consider that we do not yet know the critical target tissues involved in the toxicity of TCDD and, as noted earlier, that the relative hepatotoxic effects of TCDD in various species are not consistent. Thus, a comparison of receptors in the liver from these species may not be relevant. However, thymic atrophy is a consistent feature of TCDD exposure in all species examined. Also, results from a number of studies suggest that the relative susceptibility of a given species to TCDD-induced thymic atrophy appears to be related to the relative susceptibility to induced lethality [Table 6]. For example, the hamster is the least sensitive to both TCDD-induced thymic atrophy and lethality, while the guinea pig is the most sensitive. These data should be interpreted with some caution, as results were obtained from various laboratories utilizing different protocols and often determining the degree of thymic atrophy at different times following dosing.

[3] Gasiewicz, T. A., unpublished observations.

TABLE 6. ABILITY OF TCDD TO PRODUCE THYMIC ATROPHY IN DIFFERENT SPECIES

Species	ED_{50}(μg/kg)[a]
Guinea Pig	0.5 - 1.0[b]
Rat	15[c]
Mouse	60[d]
Hamster	> 300[e]

[a] Appropriate dose of TCDD necessary to produce 50% atrophy of the thymus.

[b] Estimated from McConnell et al., 1978. Guinea pigs sacrificed 30 days after 1,2,3,7,8-pentachlorodibenzo-p-dioxin administration.

[c] Estimated from Harris et al., 1973. Rats sacrificed at days 9 and 16 following TCDD treatment.

[d] Estimated from McConnell et al., 1978. Mice sacrificed at 30 days following TCDD administration.

[e] Estimated from Olson et al., 1980. Hamsters sacrificed at 45 days following TCDD treatment.

TABLE 7. CONCENTRATION (n) AND APPARENT DISSOCIATION CONSTANTS (K_D) OF THE THYMIC CYTOSOLIC RECEPTORS FOR TCDD IN VARIOUS SPECIES[a]

Species	n(fmol/mg protein)	K_D(nM)
Guinea Pig	47 ± 7	0.10 ± 0.07
S.D. Rat	138 ± 15	0.12 ± 0.05
Mouse:		
C57BL/6J	24 ± 2	0.27 ± 0.03
$B6D2F_1/J$	9	0.35
DBA/2J	*	*
Hamster	5 ± 6	0.24 ± 0.08

[a] As determined by hydroxylapatite assay

* Unable to be determined

With these considerations, we examined the thymus from various species for the presence of TCDD receptors. Table 7 shows the results of this study. In those species such as rat and guinea pig, which are most sensitive to TCDD-induced thymic atrophy (McConnell et al., 1978; Harris et al., 1973), the number of receptors were the highest, while hamsters, which showed the lowest or undectable levels, appeared to be least sensitive to thymic atrophy induced by TCDD (Olson et al., 1980). Additional studies (data not shown) mixing tissues and utilizing a variety of protease inhibitors showed that hamster thymus did not contain some factor(s) or protease activity which prevented us from determining, specific binding of ^{3}H-TCDD.

From these data, it is tempting to speculate that 1) TCDD-induced thymic atrophy is directly associated with the presence of receptor in that tissue, and 2) since the degree of sensitivity to TCDD-induced thymic atrophy seems to be associated with the degree of sensitivity to induced lethality, there may exist another tissue in mammals whose response to TCDD may be directly related to the presence of the receptor in this tissue and the lethal effects of this compound. However, it is clear that the relationship between the ability of TCDD to induce thymic atrophy in the whole animal and the presence of receptors is not linear. A multitude of factors may influence species susceptibility to thymic atrophy as well as the action of the receptor in this tissue. Furthermore, more direct evidence is needed to determine if there is a causal relationship between these events.

THE RELATIVE TISSUE DISTRIBUTION OF RECEPTORS FOR TCDD IN VARIOUS SPECIES

A more complete analysis of the relative tissue distribution of TCDD receptors may help to identify a particular target organ for the action of TCDD, as well as a mechanism for differential species susceptibility. In addition, and analogous to the discovery of the role of the estrogen receptor in uterine physiology, the relative tissue distribution of these proteins may be useful in suggesting a functional role of the molecule in mammals.

Table 8 shows the relative tissue concentrations of receptor for TCDD in the guinea pig and hamster as determined by the hydroxylapatite assay in our laboratory and in rats and mice as determined by Mason and Okey (1982) using the sucrose gradient method. In the guinea pig and hamster, equilibrium dissociation constants (K_D) ranged from 0.09 nM to 0.62 nM. Some interesting consistencies as well as differences were observed. In all four mammalian species examined, the liver and lung contained the highest, or nearly highest, concentrations of receptors. As noted earlier, the rat and guinea pig contain the highest level of receptor in the thymus, while lower levels were observed in mice

TABLE 8. RELATIVE TISSUE CONCENTRATIONS OF RECEPTORS FOR TCDD IN VARIOUS MAMMALIAN SPECIES[a]

Tissues	Guinea Pig[b] [Hartley Strain]	Hamster[b] [Golden Syrian]	Rat[c] [Sprague-Dawley]	Mouse[c] [C57BL/6J]
liver	59.0 ± 11.2 (8)	60.6 ± 22.5 (7)	39 ± 1.9 (8)	32 ± 1.5 (8)
lung	85.9 ± 28.0 (4)	34.5 ± 19.6 (4)	47 ± 4.3 (3)	23 ± 6.4 (3)
thymus	47 ± 7 (8)	5.1 ± 6.3 (4)	54 ± 3.9 (3)	8 ± 2.2 (5)
intestine	17.7 (2)	N.D.	15 ± 1.7 (4)	8 ± 2.3 (3)
kidney	23.9 ± 19.3 (4)	12.7 (2)	1.2 ± 0.9 (3)	10 ± 0.4 (2)
spleen	17.0 (2)	5.7 ± 5.4 (3)	---	---
testes	50.1 ± 7.2 (4)	N.D.[d]	N.D.	N.D.
heart	16.1 (2)	N.D.	N.D.	N.D.
pancreas	N.D.	N.D.	N.D.	N.D.
muscle	N.D.	N.D.	N.D.	N.D.
adrenal	N.D.	N.D.	N.D.	N.D.
prostate	---	---	---	1.3 ± 0.8 (4)
brain	---	---	N.D.	N.D.
midbrain	N.D.	N.D.	---	---
cerebrum	11.3 (2)	13.6 (1)	---	---
medulla	N.D.	N.D.	---	---
cerebellum	11.7 (2)	N.D.	---	---
hypothalamus	N.D.	N.D.	---	---

[a] Expressed as fmol/mg of cytosolic protein and mean ± S.D. of (n) determinations

[b] Gasiewicz and Rucci, unpublished observations

[c] From Mason and Okey, 1982

[d] N.D. = not detectable

and hamster. With the exception of the rat and hamster, relatively high concentrations were detected in the kidneys and intestine, respectively. Lower or undetectable levels were generally observed in other tissues. An interesting exception to this was in the guinea pig testes where exceptionally high levels of receptor were observed. No receptor has been detected in testes from any other species examined.

From these data it is difficult to make any clear correlations regarding the comparative distribution of the receptors and the relative susceptibility of various species to TCDD. The possible exception to this may be the thymus, but here it is clear that more work needs to be performed to show a causal relationship. Loss in body weight is another consistent feature of the toxicity of TCDD. As pointed out earlier in this symposium by Dr. Peterson, this effect may be mediated by alteration of hypothalamic function. Although we did not detect receptors in the hypothalamus from guinea pigs, it is possible that this effect is secondary to another tissue's response to TCDD. It is interesting, for example, that Rebar and co-workers (1982) have recently observed altered hypothalamic function by factors secreted by the thymus.

Since the receptor has been observed in many tissues such as lung and liver, where no or inconsistent toxic responses have been observed, the presence of the receptor may be essential but not necessarily sufficient for toxicity. This agrees with several observations where a number of mammalian cell lines were found to be inducible for AHH activity, suggesting the presence of receptor, but showed no toxic response following TCDD exposure (Niwa et al., 1975; Beatty et al., 1975; Knutson and Poland, 1980). It has been suggested that nearly all of the pathologic changes which occur following TCDD administration take place in epithelial tissues (Poland and Knutson, 1982). The present findings show TCDD receptors to be present primarily in epithelial tissues or those containing epithelial subpopulations. It will be of further interest to determine, for example in the thymus and lung, the particular cell type(s) with which the receptors are associated. Although the lung is not a prominent target organ for the toxicologic effects of TCDD, keratinizing squamous cell carcinoma of the lung has been observed in rats receiving chronic administration of TCDD (Kociba et al., 1978). Despite the relative inability to relate tissue receptor levels to a toxic response, and some, possibly significant, differences in tissue distribution of the receptor among species, the similarities do suggest a conservation of some functional aspect of the receptor.

THE ONTOGENY OF TCDD RECEPTORS IN MAMMALIAN SPECIES

It has become apparent over the last few years that the Ah locus is important for the induction of numerous cytochrome P-450 associated monooxygenases by certain aromatic compounds, including the halogenated dioxins. However, in various systems the interactions of these compounds with the receptor produces a much broader response including alteration of other enzyme systems which may result in cell division, involution and altered differentiation (see Table 2). It is possible that the receptor may have a role in these age-related changes during the normal development of an animal. Studies have been conducted to determine if there are age-related differences in the concentration of receptors in various species and tissues. Kahl et al. (1980) examined hepatic tissues from rat [Sprague-Dawley], mouse [C57BL/6J], rabbit [New Zealand white] and S. hispidis ["cotton rat"] for these changes. In the rat strains as well as rabbits, during the period 10 days prior to birth, the hepatic receptor concentrations were very low or undetectable. During the same time period, the hepatic receptor levels in the C57BL/6 mice were slightly higher and increased up to birth. The receptor concentrations in all four species increased postpartum, reaching peak levels between 10 to 20 days of age and then slowly declining throughout adulthood. Notably, the pattern of altered receptor concentrations with age closely matched AHH and acetanilide hydroxylase inducibility by β-naphthoflavone, as well as total cytochrome P-450 content.

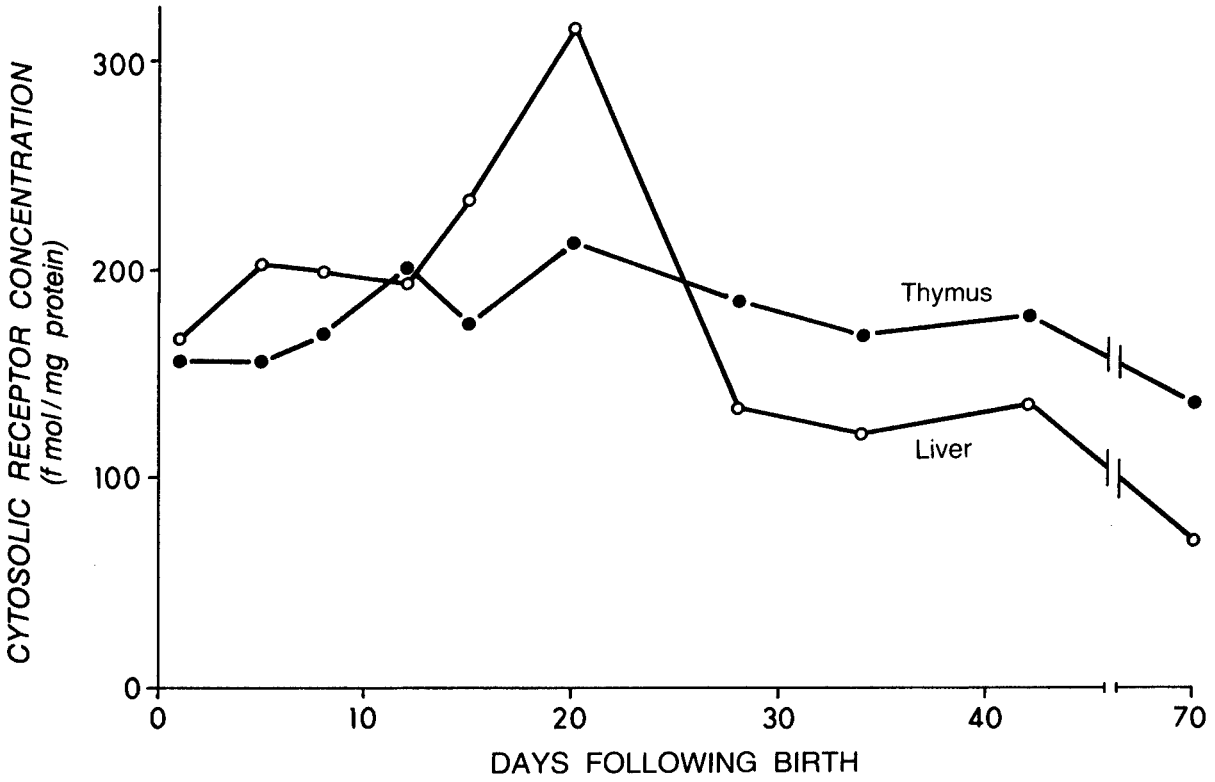

Figure 4. Hepatic and thymic cytosolic receptor levels as a function of age in Sprague-Dawley rats. Each point represents a duplicate determination of cytosol prepared by pooling tissues from 3 - 34 animals.

Recently, we performed a similar experiment in rats, examining the postpartum changes in TCDD receptor concentrations in the liver, thymus and lung [Figure 4]. The data obtained for the liver were similar to those obtained by Kahl et al.(1980) with the highest concentrations occurring between days 15 to 20. For the thymus, the concentrations were generally constant throughout the 42-day period. Similar results were observed for the lung (not shown).

In these studies, dramatic changes for many species in the concentration of these receptors in the liver appear to correspond to birth as well as with the approximate time of animal weaning (days 20 - 25). The consistency of these changes, at least in the liver, suggests a defined regulation of receptor protein.

In an additional study, Dencker and Pratt (1981) observed that in the day 13 embryo of the mouse, the concentration of receptors was higher in the maxillary process of the palate than other tissues including liver, brain, limb buds and skin. This relationship appears to correspond with the time of development of the palate as well as the ability of TCDD to cause cleft palate in this species. Thus, the maximal concentration of receptor in any particular tissue may depend on the function, if any, of that receptor in the development of that tissue.

CONCLUSIONS

It is clear from this presentation that we still do not know the exact role of the TCDD-receptor complex in the toxicity of this compound. Species and tissue specific toxicologic responses may be determined by a number of factors. In the case of inbred strains of mice, susceptibility to toxicity appears to be determined mainly by an altered receptor affinity. In other species, especially in the hamster, the number of receptors may be a significant factor in determining relative susceptibility to TCDD-induced thymic atrophy. Whether this is the case with other target tissues we do not know, simply because we do not yet know all the target tissues. A major determining factor appears to be in the species and tissue specific control over the battery of enzymes expressed or repressed. This, for example, appears to be of major significance in determining the hepatotoxic effects of TCDD in different species. An additional factor which remains to be explored is the species specific biochemical difference which may modulate the effects of altered expression.

Lastly, and perhaps the most important postulation from these data, there appears to be a conservation of some as yet unknown functional role of this receptor molecule. This evidence comes from the data showing the marked similarity of the properties of the receptor among species, the similarities in the relative tissue distribution, as well as ontogeny of the receptor. The additional observations that the main effects of TCDD appear to be on epithelial tissues and that these effects involve cell division, involution, or differentiation suggest a very basic role of this receptor in specifically epithelial tissue development. The elucidation of this function would be a major step in understanding the mechanism of TCDD toxicity, as well as the basic biochemistry of cell regulation.

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PAPER 19

TOXICITY AND CARCINOGENICITY OF POLYBROMINATED BIPHENYLS

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INTRODUCTION

In terms of volume, the halogenated biphenyls probably represent the major organic environmental contaminant (Brinkman and deKok, 1980). There can be 209 different forms (congeners) of a halogenated biphenyl depending upon the number and positions of the halogen. However, less than 50 were synthesized as commercial mixtures. Several different mixtures of polychlorinated biphenyls (PCB) were manufactured, while basically only one mixture of polybrominated biphenyls (PBB) was commercially available. Consequently, the number of PCB congeners contaminating the environment is larger than the number of PBB congeners. The mixture of PBB which contaminated the environment (DiCarlo et al., 1978) contained two congeners which account for greater than 75% of the mixture (Dannan et al., 1982). Halobiphenyls were produced commercially by the halogenation of biphenyl, and thus there is the possibility for the existence of any of the 209 congeners in any commercial mixture. This is important for several reasons: 1) the biological responses to polyhalogenated biphenyls which are of major concern are toxicity and carcinogenicity; 2) some halogenated biphenyls can be very persistent; and 3) very strict structure-activity relationships exist for all of these factors. Therefore, it is imperative that studies be conducted with pure compounds or mixtures of known composition. It is also important to determine the composition of the substances involved in human exposure.

The PCB and PBB were found to be ligands for the cytosolic receptor which avidly binds 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Poland and Glover, 1977). Although the mechanism of toxicity for the polyhalogenated aromatic hydrocarbons remains unknown, there is an excellent correlation between their toxicity and their ability to serve as ligands for this receptor (Poland et al., 1979). The polyhalogenated aromatic hydrocarbons which are capable of eliciting a TCDD-like toxicity (including TCDD) are shown in Figure 1. It is important to note that the polyhalogenated biphenyls (in this case PCB) are unique because they are the

only class in this group which has a single carbon-carbon bond between the aromatic rings. This means that the two rings can rotate essentially free of each other about the biphenyl bridge if the halogens on the rings do not interfere with rotation. This is important for the ligand binding site in the TCDD receptor appears to require a planar ligand. Only those congeners which can exist in the planar configuration, by relatively free rotation about the bridge, can serve as ligands for the TCDD receptor and elicit a TCDD-like toxicity. Thus, 3,4,5,3',4',5'-hexabromobiphenyl, which has no bromines on carbons ortho to the bridge carbons, can exist in the planar configuration, is a ligand for the receptor, and is very toxic. 2,4,5,2',4',5'-Hexabromobiphenyl, which has two bromines on carbons ortho to the bridge carbon that prohibit free rotation around the bridge and therefore planarity, shows none of the toxicities usually associated with TCDD (Render et al., 1982).

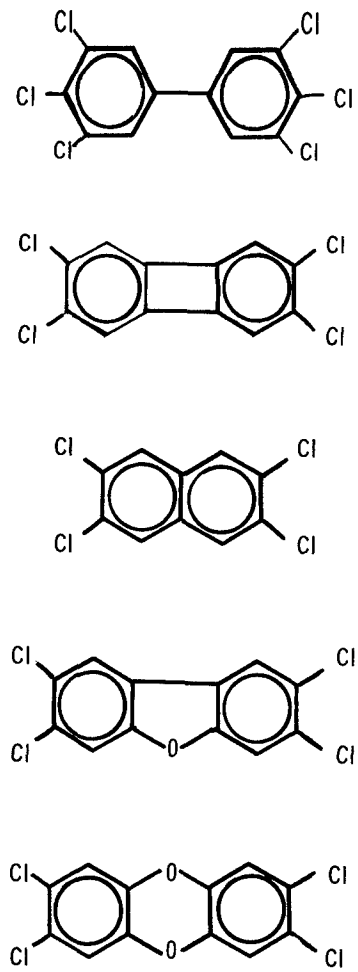

Figure 1. Structures of toxic polyhalogenated aromatic hydrocarbons.

As mentioned earlier, the mechanism by which TCDD and similar compounds cause toxicity is not known but it appears that binding by the receptor is required for toxicity. The receptor translocates the ligand to the nucleus where it brings about the induction of the synthesis of a number of enzymes. One such enzyme is a form of cytochrome P450 with arylhydrocarbon hydroxylase (AHH) activity. Thus, the ability of a compound to be a ligand for the TCDD receptor, and its potential toxicity, can be assessed by determining its ability to induce AHH activity. There is one complicating factor in this test for toxicity which is related to the metabolism of the substance in question because the toxicity elicited by these compounds is dependent upon the continued occupation of the binding site on the receptor. This is an unique feature of TCDD toxicity and may be related to the "super induction" of an enzyme regulated by the gene which responds to TCDD. Continued occupation of the receptor binding site may result in a continuous excessive level of an enzyme which finally results in toxicity (Figure 2). TCDD does not produce an immediate toxicity, no matter how high the dose. All animals die after about two weeks exposure (Neal et al., 1979). The question remains, however, as

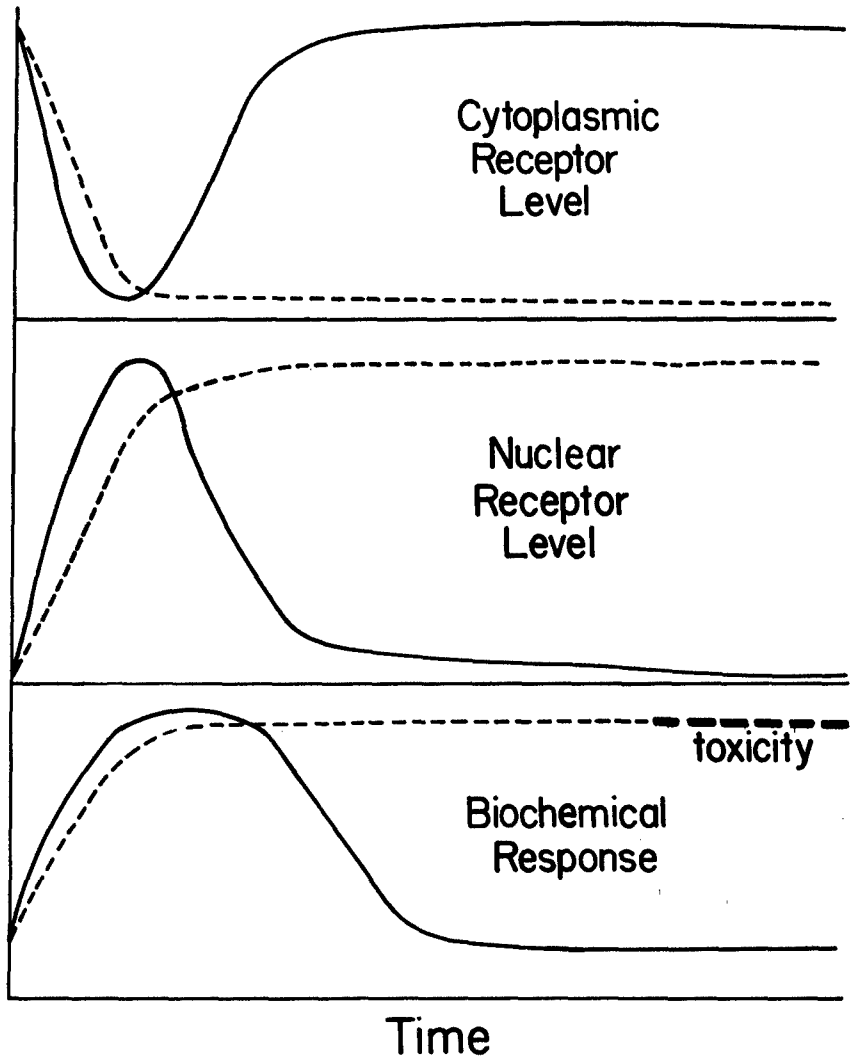

Figure 2. Possible relationships between receptor location, biochemical response and toxicity after a single dose of a persistent (----) versus non-persistent (———)polyhalogenated aromatic hydrocarbon.

to which biochemical change produces the toxicity which results in death after this period of time.

THE METABOLISM OF PBB AND ITS EFFECT ON TOXICITY

Since it is important that the toxin be bound by the receptor for a relatively long period of time, it is important to establish the rate at which the compound in question is eliminated from the body or at least converted to some form which is not a ligand for the receptor. That is, in vitro receptor binding studies may be misleading unless all of the chemicals are metabolized at the same rate (preferably close to zero).

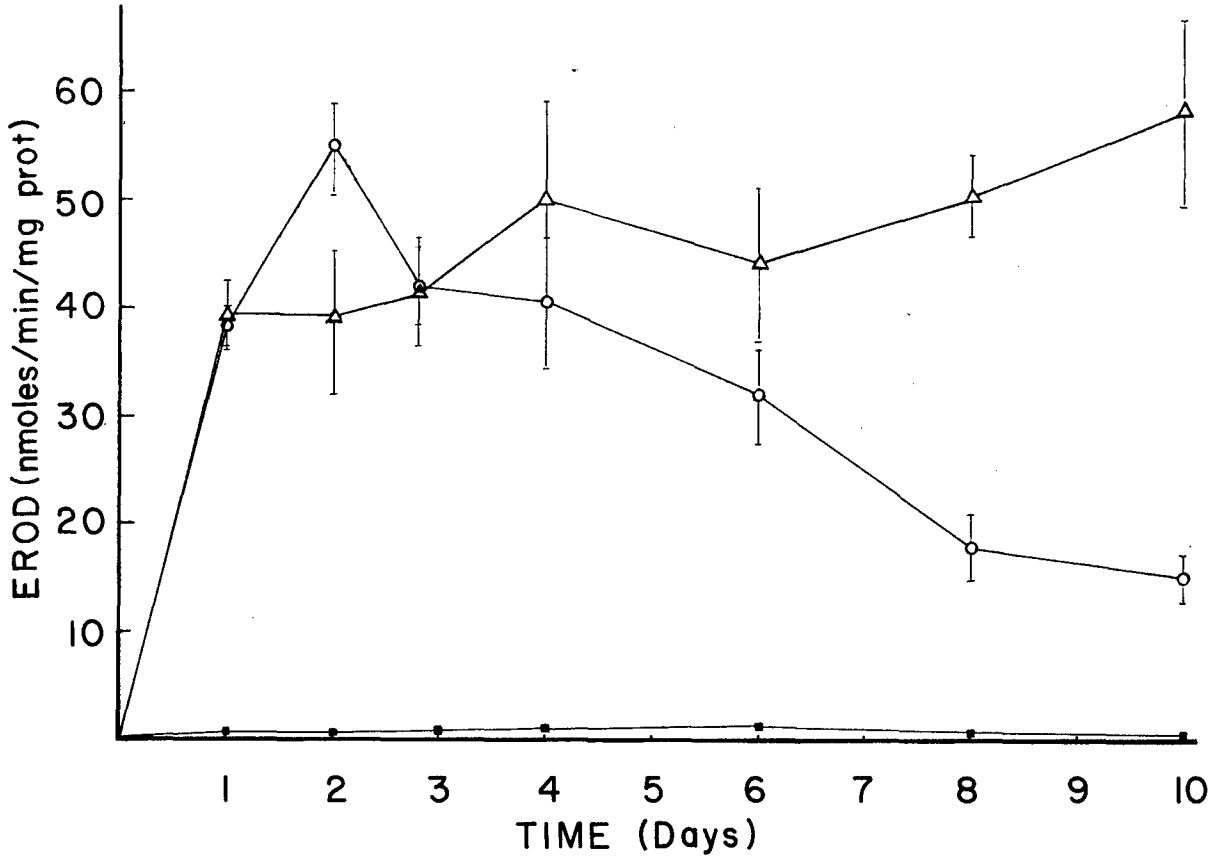

Figure 3. Ethoxyresrufin-0-de-ethylase activity in rat liver microsomes from animals treated with 21.3 μmoles/kg of either 3,4,3',4'-tetrabromobiphenyl (0) or 3,4,5,3',4',5'-hexabromobiphenyl (Δ).

Few of the PBB congeners in the mixture which contaminated Michigan are metabolized (Dannan et al., 1978). Further studies on the structure-activity relationships for metabolism with model compounds (Moore et al., 1980) revealed that two adjacent carbons must be free of bromines, and one of these must be para to a bridge carbon in order for a PBB congener to be metabolized by a mixed-function oxidase induced by phenobarbital. In addition, the greater the number of bromines on carbons ortho to the bridge carbon, the greater the rate of metabolism, if all of the requirements listed above are satisfied. Further studies with toxic congeners have revealed how metabolism can influence toxicity. In this experiment, rats were given a single equal molar dose (21.3 μmoles/kg body weight) of either 3,4,5,3',4',5'-hexabromobiphenyl or 3,4,3',4'-tetrabromobiphenyl and killed at various times up to ten days after treatment. Liver microsomes were isolated and assessed for AHH induction (ethoxyresrufin-0-de-ethylase, EROD activity) (Figure 3). In addition, tissues were extracted and

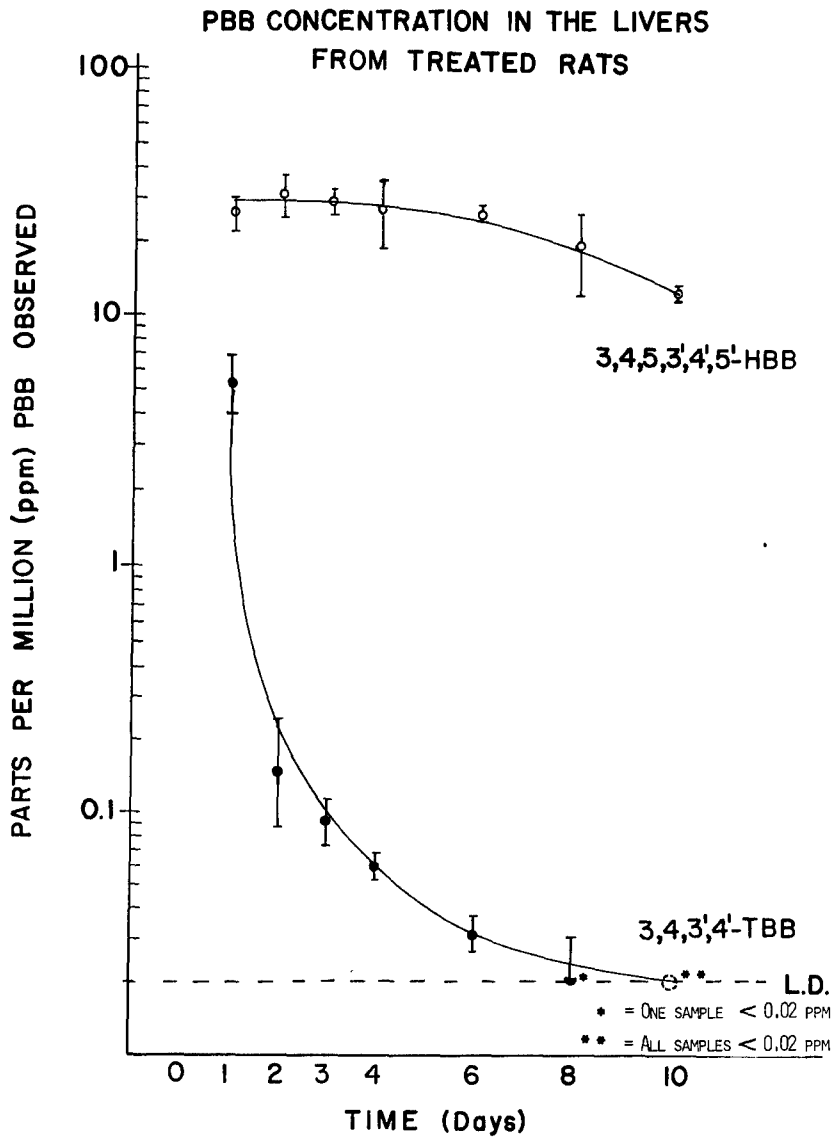

Figure 4. Concentration of 3,4,3',4'-tetrabromobiphenyl or 3,4,5,3',4',5'-hexabromobiphenyl in the livers of rats treated with 21.3 μmoles/kg of the respective congeners.

assayed for the respective treatment compounds by gas chromatography (Figure 4). The results show that while 3,4,5,3',4',5'-hexabromobiphenyl and 3,4,3',4'-tetrabromobiphenyl caused about the same level of enzyme induction, only the former caused sustained induction. Tissue levels basically followed EROD activity in that the level of the hexabromobiphenyl remained high throughout the time period, whereas the tissue levels of the tetrabromobiphenyl decayed exponentially. Further experiments with a number of model congeners and with liver microsomes from animals pretreated with a variety of microsomal enzyme inducers indicated

that a form of cytochrome P450 induced by 3-methylcholanthrene (3-MC) was responsible for the metabolism of the 3,4,3',4'-tetrabromobiphenyl. The enzyme induced by 3-MC would only metabolize congeners with four or less bromines. In addition, adjacent carbons ortho and meta to a bridge carbon must be free of bromine. Thus, the following compounds were metabolized by microsomes from animals pre-treated with 3-MC: 3,4,3',4'-, 2,3,3',4'-, 2,5,3,4'-, 2,4,2',5'-, and 3,4,3',5'-tetrabromobiphenyls. It should also be noted that the toxic PBB congeners induce the same enzymes induced by 3-MC (Dannan et al., 1982); therefore, these compounds can induce their own metabolism if they satisfy the structural requirements for toxicity and metabolism.

The structure-activity relationships for the induction of liver microsomal mixed-function oxidases by the halogenated biphenyls are more complicated than those for induction by other polyhalogenated aromatic hydrocarbons shown in Figure 1, such as the dibenzo-p-dioxins. In this case, dioxins only appear to induce those enzymes induced by 3-MC. However, certain PBB or PCB congeners induce enzymes which are also induced by phenobarbital (Parkinson and Safe, 1981). The induction of liver microsomal enzymes which are induced by a combination of 3-MC and phenobarbital is often termed a mixed-type induction. It was observed by Dent and co-workers (Dent et al., 1976a,b) in studies with the PBB mixture but it was subsequently observed for a pure PBB congener by Dannan and collaborators (Dannan et al., 1978). There have subsequently been numerous reports of mixed-type induction by PCB and PBB congeners (Parkinson and Safe, 1981; Goldstein, 1979). Four such congeners have been found in the PBB mixture which contaminated Michigan (Dannan et al., 1978; Dannan et al., 1982; Dannan et al., In Press). The relative effectiveness for the induction of one or the other type can vary for the different congeners (Dannan et al., In press).

THE CARCINOGENIC POTENTIAL OF PBB

The PBB mixture which contaminated Michigan has also been found to be carcinogenic in rats (Kimbrough et al., 1981). Since there have been no reports demonstrating the mutagenic potential of the polyhalogenated aromatic hydrocarbons, Pitot (1980) and others have postulated that these compounds may cause hepatic cancer by promotion of environmentally initiated cells. Promoters appear to cause a variety of hormone-like cellular responses (Slaga et al., 1978; Coburn, 1980), and Trosko has proposed that promotors also inhibit metabolic cooperation (Yotti et al., 1979). Elimination of metabolic cooperativity (cell-cell communication) would allow the proliferation of clones of initiated cells (Trosko and Chang, 1980). PBB congeners which inhibit metabolic cooperativity (Tsushimoto et al., 1982) should also be positive in the tumor promotion assay of Pitot (1978).

Both the toxic and the non-toxic congeners were found to be positive in the promotion test, albeit at quite different doses (Table 1). The toxic congener was found to be a very effective promoter in the range of 1 ppm in the diet whereas the non-toxic congener was about equally effective at 10 ppm. Both congeners gave a linear dose response. The non-toxic congener used in this study does not induce AHH activity nor does it cause any of the pathological changes usually observed in animals treated with the toxic congeners (Moore et al., 1978). However, this congener is a very effective phenobarbital-type inducer. It was very interesting to note that when this congener was fed along with 500 ppm phenobarbital there seemed to be an additional number of enzyme-altered foci.

TABLE 1. TUMOR PROMOTION BY PBB[a] THIRTY DAYS AFTER PARTIAL HEPATECTOMY AND 10 mg/kg DIETHYLNITROSAMINE

	Treatment (140 days)	Dose		Foci/cm^3
	None			198
	Phenobarbital	500	ppm	1220
	Firemaster	10	ppm	1523
	Firemaster	100	ppm	415
	2,4,5,2',4',5'-HBB	10	ppm	1227
	2,4,5,2',4',5'-HBB	100	ppm	2195
	3,4,5,3',4',5'-HBB	0.01	ppm	233
	3,4,5,3',4',5'-HBB	0.1	ppm	268
	3,4,5,3',4',5'-HBB	1.0	ppm	1005
	2,4,5,2',4',5'-HBB	10	ppm	
+	3,4,5,3',4',5'-HBB	0.1	ppm	3116
	2,4,5,2',4',5'-HBB	100	ppm	
+	3,4,5,3',4',5'-HBB	1	ppm	489
	2,4,5,2',4',5'-HBB	10	ppm	
+	Phenobarbital	500	ppm	1819

[a] Female rats were fed diets containing the indicated levels of compounds for 140 days thirty days after partial hepatectomy and 10 mg/kg diethylnitrosamine.

The non-toxic congener was also shown to inhibit metabolic cooperativity; however, the toxic congeners always affected cell survival before significant inhibition of metabolic cooperativity could be demonstrated (Tsushimoto et al., 1982). However, cell killing could be viewed as the inhibition, or at least the prevention, of metabolic cooperativity. Rats were fed 0.01, 0.1, or 1.0 ppm 3,4,5,3',4',5'-hexabromobiphenyl for 140 days in the Pitot test for promotion and the liver assessed for pathological effects, induction of AHH activity, and γ-glutamyl transpeptidase positive foci. AHH induction increased linearly with dose but the number of enzyme-altered foci was significantly increased only in the liver of animals treated with sufficient chemical (1.0 ppm) to cause significant histologic and histochemical changes (Table 2) (Jensen et al., In Press).

TABLE 2. A COMPARISON OF HISTOLOGICAL CHANGES, AHH ACTIVITY AND THE NUMBER OF ENZYME ALTERED FOCI IN LIVERS OF RATS TREATED WITH 3,4,5,3',4',5'-HEXABROMOBIPHENYL (HBB)[a]

Treatment (Dose)	Histological Changes	AHH Activity[b]	Enzyme altered Foci/cm^3
None	---	0.3	198
Phenobarbital (500 ppm)	---	0.4	1220
3,4,5,3',4',5'-HBB (0.01 ppm)	---	1.8	233
3,4,5,3',4',5'-HBB (0.1 ppm)	---	10.8	268
3,4,5,3',4',5'-HBB (1.0 ppm)	+++	23.4	1005

[a] Female rats were fed a diet containing the indicated amount of compounds for 140 days thirty days after partial hepatectomy and 10 mg/kg diethylnitrosamine.

[b] Ethoxyresrufin-0-de-ethylase activity, nmoles/mg protein/min.

The dose-response curve for the promotion of liver enzyme altered foci by the PBB mixture which contaminated Michigan (Firemaster) has never been found to be linear. This mixture is made up of about twelve major components, four of which probably account for almost all of the toxicity of the mixture (Dannan et al., In Press). The rest of the mixture is made up of strictly phenobarbital-type inducers. Experiments in which animals were simultaneously treated with both a toxic and a non-toxic congener showed that one of the compounds must have been able to inhibit the ability of the other to increase the number of enzyme altered foci (Table 1). It might be very reasonable to propose that a toxic congener might inhibit the ability of a non-toxic congener

to promote, by inhibition of enzyme induction for example, but the reverse is difficult to explain. That is, promotion by a toxic congener should not be decreased by a non-toxic congener. At the very least, these data suggest two different mechanisms of promotion and that promotion by the toxic congener does not correspond to AHH induction. AHH induction may be required but it is not sufficient to promote the activity of diethylnitrosamine. Whether induction of other enzymes by the non-toxic congeners is involved in promotion remains to be determined.

RELATIONSHIP BETWEEN TOXICITY AND VITAMIN A STATUS

One additional factor which may be extremely important in both the toxicity and carcinogenicity of PBB or other polyhalogenated aromatic hydrocarbons is the vitamin A status. Interestingly there are many similarities between the toxic manifestations of polyhalogenated aromatic hydrocarbons and the symptoms of vitamin A deficiency, as pointed out by Thunberg and associates (1980). These include decreased growth rates, keratinization or atrophy of epithelial cells, acne, and immunosuppression. In addition, vitamin A has been related to carcinogenesis in that hypovitaminosis A has been associated with increased incidence of human lung cancer (Bjelke, 1975; Basu et al., 1976) and stomach cancer (Abels et al., 1941; Basu et al., 1974). On the other hand, the ability of vitamin A and its analogues to affect tumor promotion in skin and to prevent epithelial cancer has also been reported (for a review see Sporn, 1977). Vitamin A and its analogues were found to be potent inhibitors of the tumor promoting activity of phorbol acetate (Verma et al., 1978).

Thunberg and co-workers first showed the effect of a single dose of TCDD on the status of vitamin A in the rat (Thunberg et al., 1979). The storage of vitamin A in the liver decreased with time to about 30% of control level. PCB were reported to cause decreased levels of liver vitamin A in Japanese quail and rats in 1973 (Cecil et al., 1973). Vitamin A levels were also shown to be depressed in cattle with hyperkeratosis (Olafson, 1947), a disease later shown to be caused by chlorinated naphthalenes (Sikes and Bridges, 1956). Vitamin A storage in the fetal liver was decreased by the administration of high doses of PCB (Villeneuve et al., 1971). Rats fed a diet containing 0.1% PCB supplemented with 3,400 IU of vitamin A grew better than those fed the 0.1% PCB diet without supplementary vitamin A (Innami et al., 1974). Serum retinyl palmitate levels were not affected in animals treated with PBB but serum retinol levels were drastically reduced (Darjono, 1982). Rats fed 100 ppm PBB lost less body weight when their diet contained 30,000 IU of vitamin A palmitate/kg feed (Figure 5). Thymic weights were approximately normal when the animals were fed the diet containing excess vitamin A (Table 3). Serum vitamin A levels were altered by PBB and were greatly influenced by dietary vitamin A (Table 4).

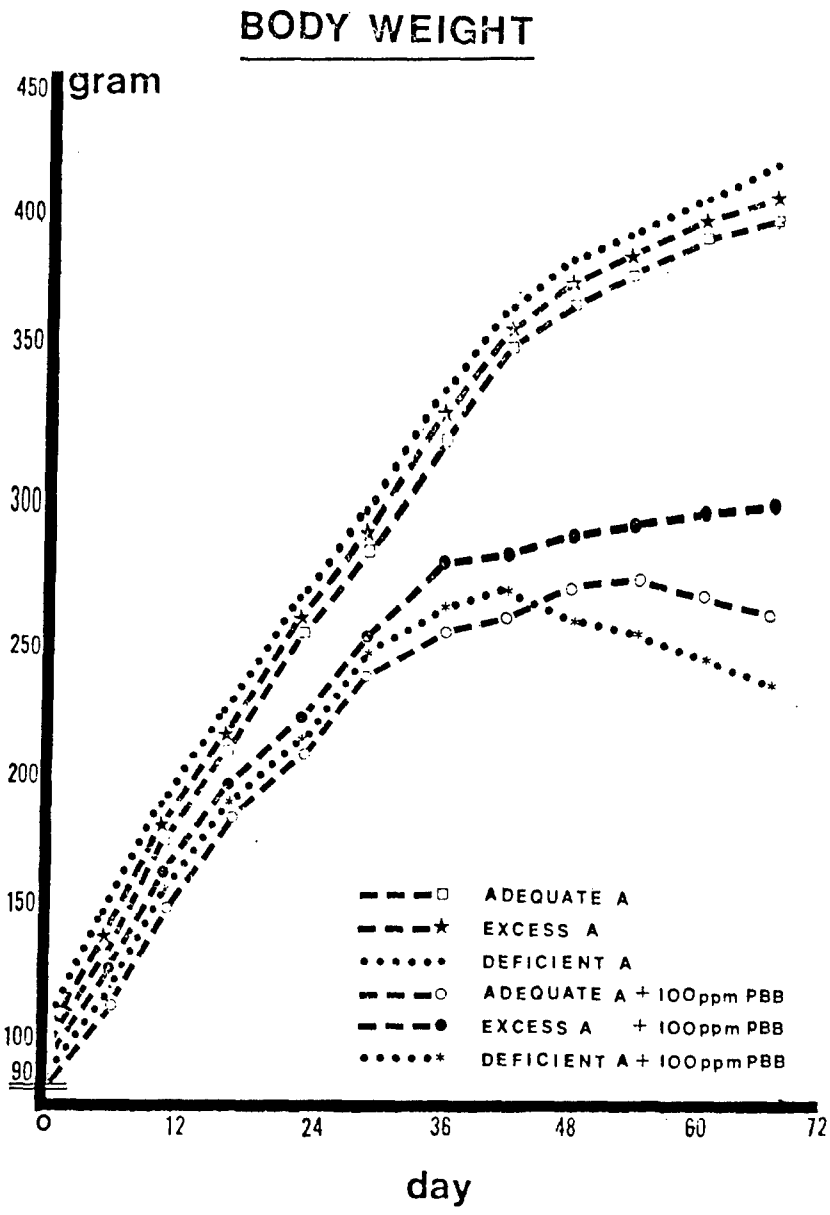

Figure 5. Means of body weight of rats fed diet adequate, excess or deficient in vitamin A with or without PBB.

The vitamin A status of an animal must be evaluated in terms of total vitamin A in the diet, total vitamin A in the serum, and total vitamin A in the liver. The total vitamin A in the serum depends on the total vitamin A in the diet and in the liver. Under normal conditions, blood levels of vitamin A are maintained within a relatively narrow range, influenced only in part by dietary intake of the vitamin as long as some reserve supply remains in the liver (Keilson et al., 1979). Plasma levels in rats fed vitamin A deficient diets remained above 300 ng/ml until liver reserves were below 10 µg/g (Muto et al., 1972).

TABLE 3. THYMIC WEIGHTS OF RATS FED VITAMIN A ADEQUATE, EXCESS, OR DEFICIENT DIETS CONTAINING 0 OR 100 ppm PBB FOR 66 DAYS (g/100g Body Weight, Mean ± Standard Deviation)

Vitamin A	PBB 0	PBB 100 ppm
Excess	0.20 ± 0.03	0.18 ± 0.03
Adequate	0.22 ± 0.04	0.13 ± 0.02
Deficient	0.20 ± 0.03	0.09 ± 0.03

* * * * *

TABLE 4. VITAMIN A CONCENTRATIONS IN SERA FROM RATS FED VITAMIN A ADEQUATE, EXCESS, OR DEFICIENT DIETS CONTAINING 0 AND 100 ppm PBB FOR 66 DAYS (ng/ml, Mean ± Standard Deviation)

Vitamin A	PBB 0 ppm	PBB 100 ppm
Excess Diet		
Retinyl Palmitate	44.2 ± 7.7	47.5 ± 7.6
Retinol	411.9 ± 18.7	241.5 ± 34.5
Total	456.1 ± 22.2	289.0 ± 34.0
Adequate Diet		
Retinyl Palmitate	40.0 ± 0.6	39.7 ± 7.4
Retinol	234.1 ± 22.7	91.0 ± 19.1
Total	274.1 ± 18.6	130.7 ± 24.6
Deficient Diet		
Retinyl Palmitate	41.0 ± 13.5	44.8 ± 9.6
Retinol	82.7 ± 18.6	5.7 ± 4.5
Total	123.7 ± 19.6	51.5 ± 11.0

The combined effect of PBB and low vitamin A in the diet produced a significant decrease in total vitamin A in the sera. Since the value for retinyl palmitate was almost constant from one group to the other (deficient, adequate, and excess vitamin A),

the difference in total vitamin A levels among the treatment groups as produced by the difference in the retinol fraction. Normally there are only two forms of vitamin A in the liver of rats, retinyl palmitate and a small amount of retinol. In the animals treated with PBB there was also retinyl acetate in the liver. The presence of retinyl acetate seemed to be related to the disappearance of the retinol fraction and the fact that the vitamin A reserve had fallen below the critical point. It was also related to the appearance of the decrease in retinol concentration in the serum. It would appear that PBB may have affected the retinol esterification (and hydrolysis) process. This possibility must be investigated. It would appear that there is no altered glucuronidation of retinol but there is evidence of excess oxidation of vitamin A. Also, it will be important to study the relationships between toxicity and the various forms of vitamin A.

The pathological effects of PBB were also dramatically affected by vitamin A status, the most striking being massive enlargement of the common bile duct in rats fed a diet deficient in vitamin A and containing high levels of PBB (Darjono, 1982). The lesions consisted of extensive hyperplasia and resembled a preneoplastic state. Also, rats fed a vitamin A deficient diet containing 100 ppm PBB had the lowest thymus weights and the rats fed the diet containing excess vitamin A and 100 ppm PBB had the same thymic weights as animals fed the excess vitamin A without PBB. Interestingly, there were no differences in AHH activity in the liver microsomes from animals treated with PBB and the different levels of vitamin A. It will be imperative to assess the effect of dietary vitamin A status on the tumor promotion effects of PBB.

SUMMARY

The different PBB congeners are in fact very different chemicals with different biological responses. Some are metabolized by liver microsomal enzymes which are induced by phenobarbital. Some are metabolized by enzymes induced by 3-MC. Some of the PBB congeners induce enzymes which are induced by phenobarbital, some induce enzymes induced by 3-MC, and some induce both types of enzymes. The congeners which induce these enzymes aren't necessarily metabolized. The toxic congeners must be able to exist in a planar configuration, apparently in order to serve as a ligand for the TCDD receptor, because toxicity is well correlated with the induction of AHH activity. Both toxic and non-toxic congeners appear to promote the development of enzyme altered foci in rats treated with diethylnitrosamine. Promotion by a toxic congener does not seem to be correlated with liver microsomal enzyme induction but appears related to pathology. The toxic and non-toxic congeners do not appear to be promotors in the same dose range, and perhaps not by the same mechanisms.

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PAPER NO. 20

THE TOXICOLOGY OF PERFLUORODECANOIC ACID IN RODENTS

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Perfluorinated compounds have wide application. Perfluorocarboxylic and perfluorosulfonic acids are used commercially in plating systems, as wetting agents, and as corrosion inhibitors. Seven to nine carbon perfluorinated fatty acid compounds are used to impart water and oil resistance to paper, fabrics, and leather (Rozner and Taves, 1980). Many perfluorinated organic liquids are synthesized for industrial use as heat exchangers, leak detectors, and hydraulic fluids because they are electrically non-conductive, chemically non-reactive, and heat stable (Clark et al., 1973). Aqueous film-forming foams, used as fire extinguishants, contain commercial mixtures of derivatized perfluorinated fatty acids. The use of perfluorinated compounds for vascular fluid replacement has been advocated due to "the virtual lack of toxicity of these materials coupled with their high gas solubility capacities" (Sargent and Seffl, 1970). Perfluorinated organic liquids have also been proposed for use as computer-assisted tomography (CAT) scan media (Enzymann and Young, 1979).

Perfluoro-n-decanoic acid or nonadecafluoro-n-decanoic acid (NDFDA) is a straight chain, perfluorinated, ten-carbon acid ($CF_3(CF_2)_8CO_2H$) with formula weight of 514.08. It has a melting point of 77° - 79°C, boiling point of 218°C at 740 mm Hg pressure, and is readily obtainable in crystalline form.[1] While investigating the toxicity of aqueous film-forming foams, Naval personnel compared the effects of various perfluorinated fatty acids,

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including NDFDA and the eight-carbon perfluorinated analog perfluorooctanoic acid or PFOA. The only observable effect following intraperitoneal injection of PFOA at a dose of 100 mg/kg in young adult male Fischer 344 rats was a transient decrease in body weight. Recovery appeared to be complete within one week. At intraperitoneal or oral doses of NDFDA of 100 mg/kg, rats became severely anorectic, had severe weight loss, and died within two to three weeks of treatment.

Following these initial studies, additional work has been accomplished at the Toxic Hazards Division, Air Force Aerospace Medical Research Laboratory. Experiments were conducted to determine the LD_{50} and to evaluate survival times of rats after single intraperitoneal (IP) injections of NDFDA or of PFOA, the shorter-chain analog, dissolved in propylene glycol/water (1:1, v:v). Ten male Fischer 344 rats were used in each dose group and an additional 10 rats were given 2 mL/kg propylene glycol/water, the same dosing volume used with all experimental animals. With NDFDA, the $LD_{50/14}$ days was 63.6 mg/kg, and the $LD_{50/30}$ days was 41.4 mg/kg. With PFOA, there was no mortality after the 5th day following injection, and both the $LD_{50/14}$ and $LD_{50/30}$ were 188.7 mg/kg.

When mean survival time is plotted versus dose for NDFDA and PFOA (Figure 1), significant differences are readily noted. Even though the curves are very similar at high doses, at doses below 175 mg/kg they vary significantly. Delayed mortality is not observed with PFOA but is a hallmark of the acute IP toxicity of NDFDA in the dose range of about 40 - 150 mg/kg. As noted earlier, this delayed toxicity of NDFDA is characterized by marked reduction in food intake and loss of weight (Figure 2). Over a 2- to 3-week period following a single 50 mg/kg IP dose, rats lose nearly half their body weight. In addition, during a 7- to 10-day period, from about the 6th day on, treated rats eat essentially no food at all. Water intake does not differ from that of pair-fed controls. Blood counts, serum electrolytes, and serum transaminase levels measured at 8 days post-exposure do not appear to be significantly different from those of pair-fed controls (Table 1).

Much more limited work has been done to characterize the acute toxicity of single doses of NDFDA in other rodent species. The species used in these more restricted studies were mouse, guinea pig, and hamster. Hamsters respond in a manner very similar to that of rats. There is a reduction in food intake (Figure 3), but not as marked as that occurring with the rats, and a loss of body weight which is not reversed until 50 - 70 days following treatment (Figure 4). The LD_{50} is somewhere between 50 and 100 mg/kg and deaths are observed as late as the 27th day after injection. The LD_{50} appears to be slightly higher in mice and guinea pigs and recovery from the single dose begins much sooner. With these two species, most deaths occur within the first week.

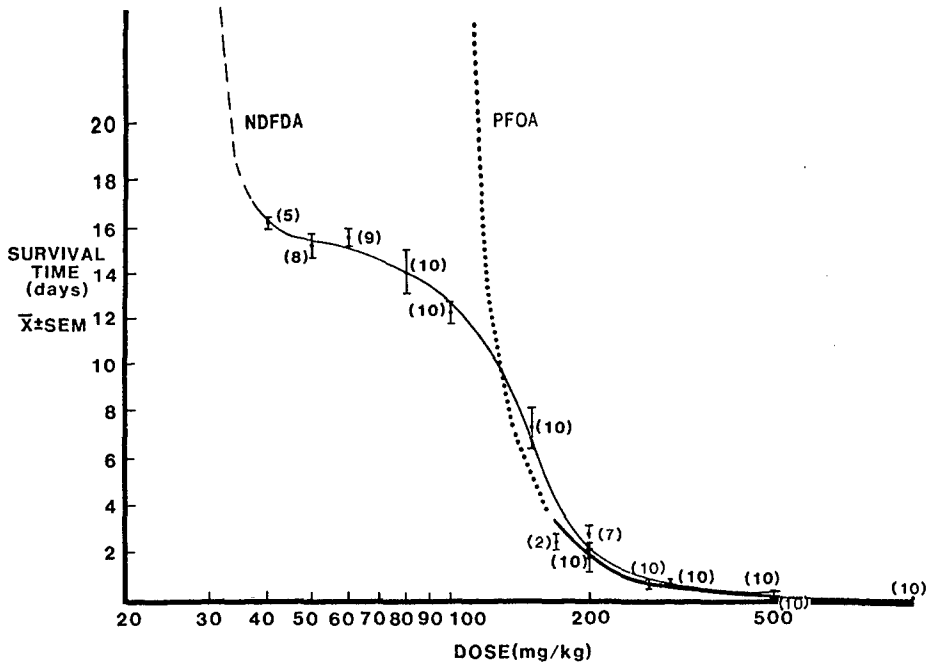


Figure 1. Mean survival times after IP injection of PFOA and NDFDA. In parentheses after plotted points are the number of animals responding.

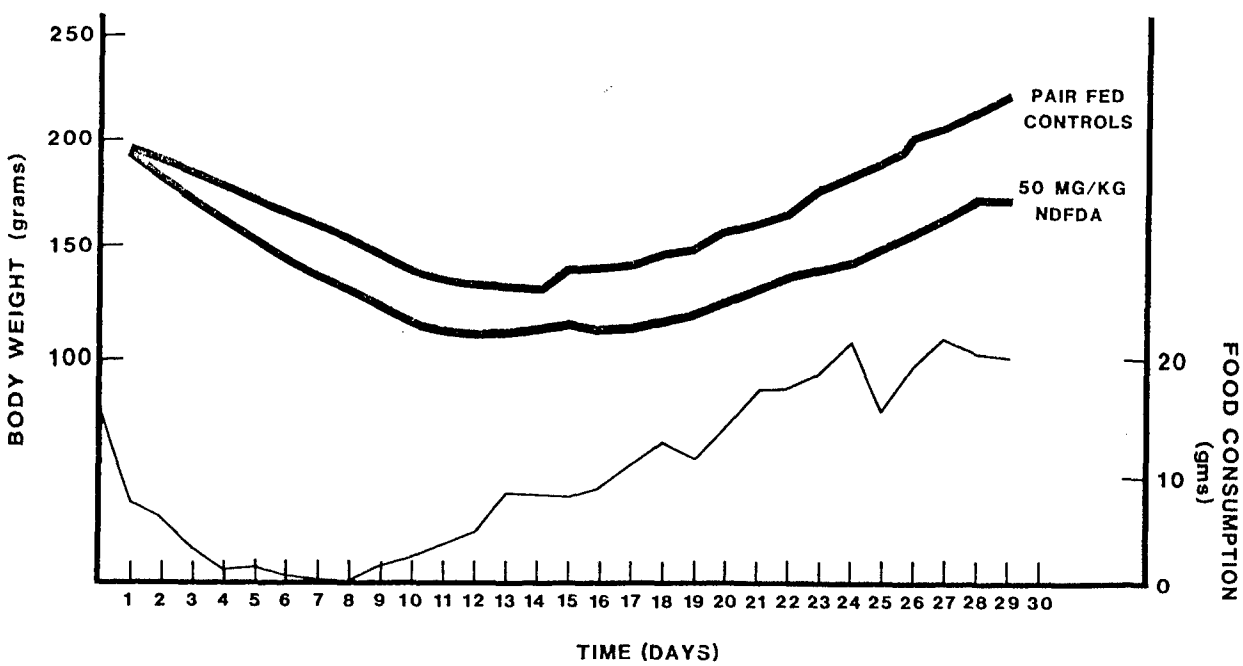


Figure 2. Mean body weights and food consumption of male Fischer 344 rats given 50 mg NDFDA/kg IP.

TABLE 1. DETERMINATION OF SELECTED HEMATOLOGY AND CLINICAL CHEMISTRY PARAMETERS IN MALE RATS 8 DAYS AFTER A SINGLE INTRAPERITONEAL INJECTION OF 50 MG OF NDFDA/KG

	Pair-Fed Control	NDFDA
Hematology:		
RBC (x 10^6/mm^3)	9.31 ± 0.28[a]	10.23 ± 0.52
HCT (vol. %)	51.6 ± 1.0	59.8 ± 2.7
HGB (g/dl)	17.4 ± 0.6	19.2 ± 0.8
MCV (cμ)	55.3 ± 1.1	58.4 ± 1.1
MCH (μμg)	18.7 ± 0.2	18.7 ± 0.4
MCHC (%)	33.8 ± 0.9	32.1 ± 0.6
WBC (x 10^3/mm^3)	5.2 ± 1.1	7.3 ± 2.0
Neutrophil (%)	16.1	25.5
Lymphocyte (%)	82.3	69.6
Eosinophil (%)	1.0	1.0
Monocyte (%)	1.2	1.5
Clinical Chemistry:		
K (meq/liter)	6.3 ± 0.8	5.7 ± 0.7
Na (meq/liter)	152 1 ± 3.7	150.3 ± 4.8
Cl (meq/liter)	112.6 ± 2.1	113.9 ± 2.6
SGOT (units/liter)	115.8 ± 18.9 (n=9)	129 9 ± 29.5 (n=7)
SGPT (units/liter)	68.4 ± 16.7 (n=9)	42.6 ± 16.9 (n=7)
Bilirubin (mg/dl)	0.1 ± 0.05 (n=7)	0.1 ± 0.06 (n=6)

[a] Values are mean ± standard deviation with n=10.

At the light microscopic level, pathologic effects associated with a single dose of NDFDA were found in thymus, bone marrow, stomach, mesentery, liver, and testes of the rat. The first histologic lesion in the thymus was hemorrhage at two days postexposure and by the eighth day atrophy was histologically apparent. Bone marrow depletion, involving all cell types, was evident by day four. Thymus and marrow recovered normal architecture in surviving animals by day 36. Acute gastric and mesenteric edema was observed by the eighth day. An alteration in hepatic morphology was evident by the second day, with bile duct proliferation present by the 16th day. Early degenerative testicular changes were seen at day eight and atrophy and necrosis were pronounced by

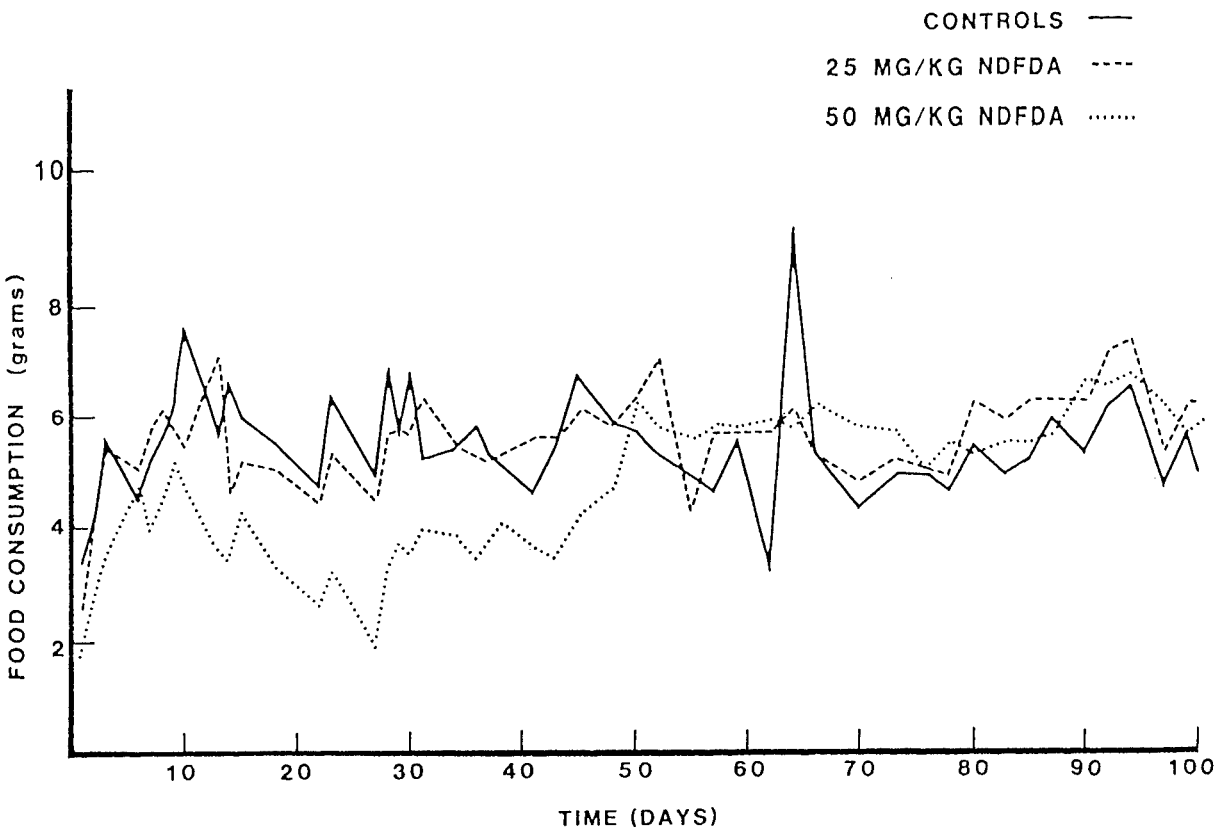


Figure 3. Average food consumption in hamsters after IP injection of NDFDA.

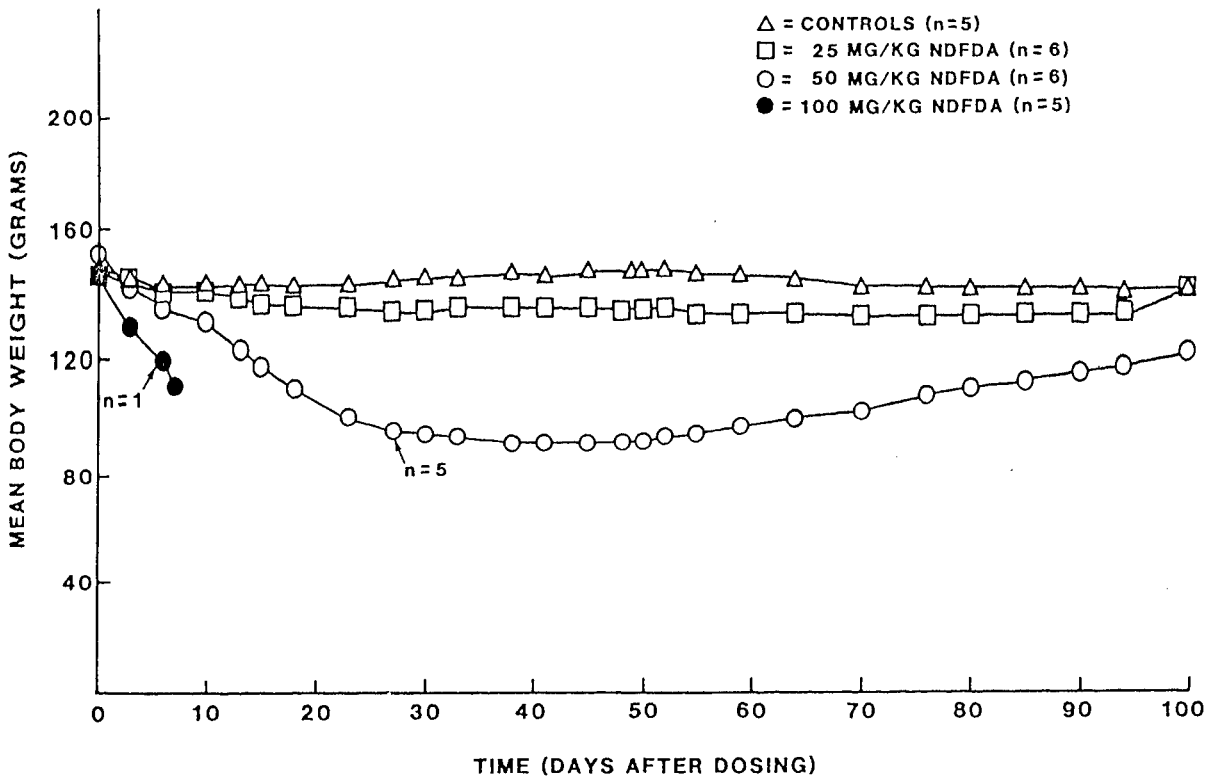


Figure 4. Mean body weight of hamsters after IP injection of NDFDA.

the 16th day (Figure 5). The effects in the testes seem to take a time course in which mild, diffuse atrophy is followed by interstitial hemorrhage and hyperemia, and then by frank necrosis and calcification.

Electron microscopic evaluations have been conducted on livers from the four rodent species used in acute toxicity studies. Extensive ultra-structural changes were noted in all four species, but the nature of the alterations varied somewhat from species to species. The major effects common to all species were loss of normal architecture of mitochondria and rough endoplasmic reticulum, and a marked increase in the number of peroxisomes. These changes in a rat liver 16 days after dosing are evident in Figure 6. Peroxisome proliferation was greatest in mouse liver (Figure 7) and least in the guinea pig liver (Figure 8). Guinea pig livers showed the most extensive disruption of normal hepatic structure. Numerous fatty vacuoles were found in both the guinea pig and hamster livers (Figure 9). Van Rafelghem has suggested that the differences in appearance of the livers in the four species may be more related to different patterns of food intake than qualitative differences in toxicity. For instance, there is no fatty infiltration in rat liver at day 16, while there is in hamsters. Yet, rats have been essentially fasted for a week or ten days at this time. The hamsters have reduced food intake, but do continue eating at about 60% of the control level. Rats might also show fatty vacuolization if they were force-fed during the two weeks after NDFDA administration. This study is being pursued in our laboratory.

Explanation of Figures

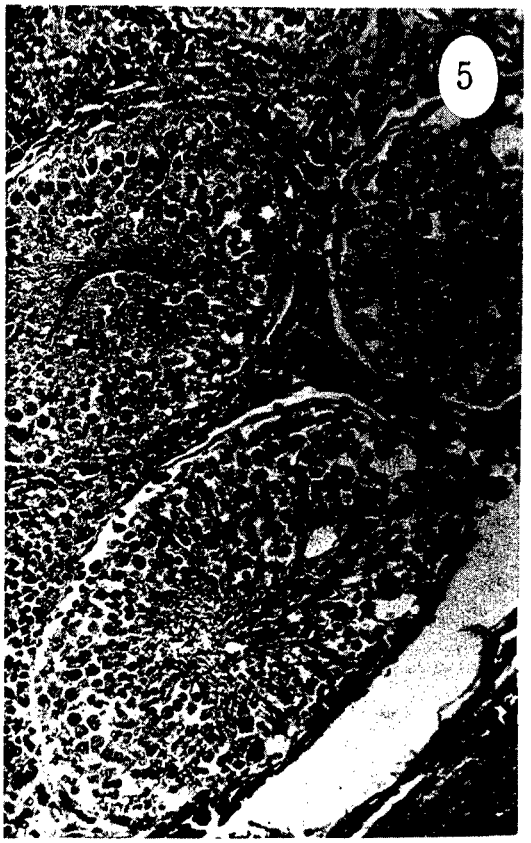
Figures 5 - 8

Figure 5. Rat testis 16 days after animal given 50 mg NDFDA/kg ip. X 12,000.

Figure 6. Rat liver 16 days after animal given 50 mg NDFDA/kg ip. X 12,000.

Figure 7. Mouse liver 28 days after animal given 150 mg NDFDA/kg ip. X 12,000.

Figure 8. Guinea pig liver 14 days after animal given 175 mg NDFDA/kg ip. X 12,000.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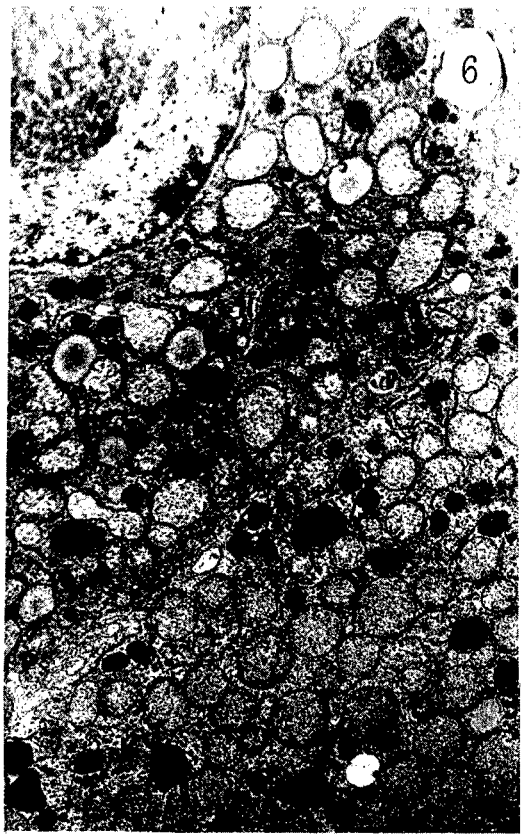
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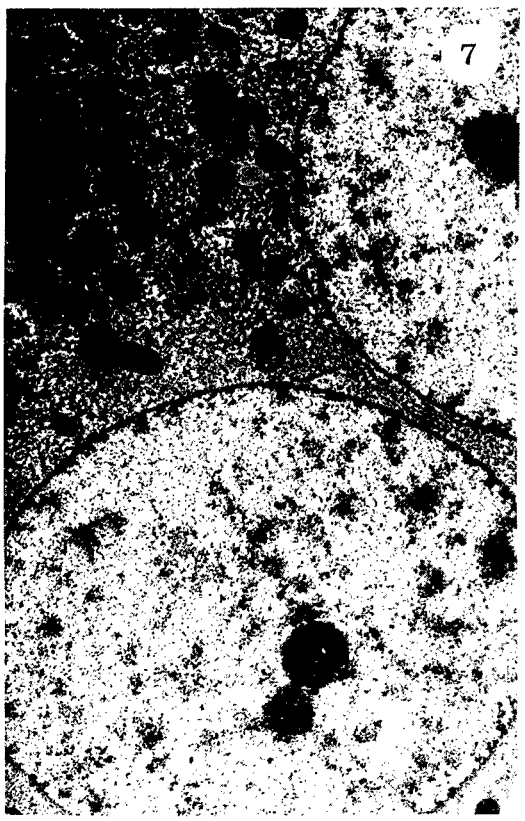
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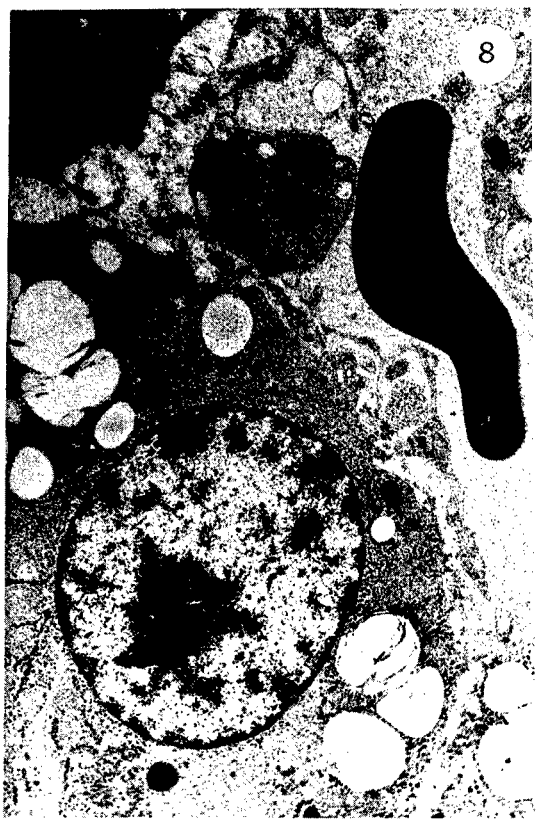
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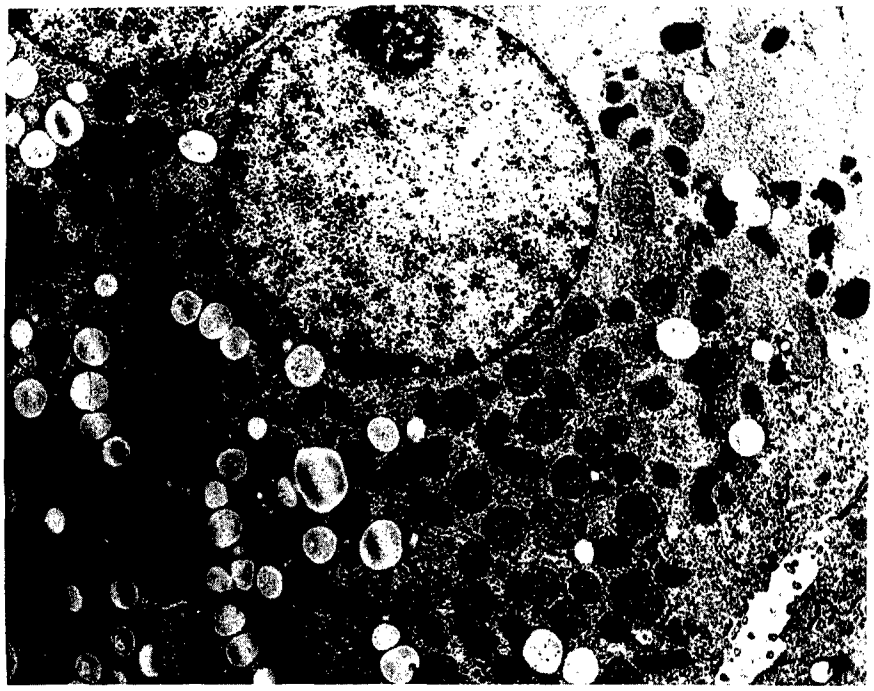

Figure 9. Hamster liver 16 days after animal given 50 mg NDFDA/kg ip. X 12,000.

Because of the marked increase in peroxisomes, which are thought to be important in fatty acid metabolism, especially fatty acid oxidation, and because the chemical of interest is an analog of a naturally occurring short chain fatty acid, and because the liver was so profoundly affected in all species by NDFDA, we determined the distribution of various fatty acids in livers of treated rats at various times after treatment. Studies were also conducted for testes and blood fatty acids (Olson, 1982), but the liver was the organ most markedly altered and is the only one discussed here. Four groups of rats were used in these studies. Group I received 50 mg NDFDA/kg; Group II received 100 mg PFOA/kg; Group III received 2 ml propylene glycol:water/kg and were fed ad libitum. Each control rat of Group IV received 2 ml propylene glycol:water/kg and was pair-fed with a matching NDFDA-exposed rat of Group I to define the effect of decreased food intake. Rats were killed 2, 4, 8, 16, and 30 days after injection. Fatty acids were obtained by homogenization of liver samples in chloroform-methanol (2:1), filtration, and isolation of the chloroform-soluble portion. They were esterified by refluxing in benzene and 3N methanolic-HCl and detection was accomplished by FID gas chromatography. To assure proper identification of peaks, reference standards were injected on the column.

The relative amounts of the six major fatty acids - palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), arachidonic (20:4), and docosahexaenoic (22:6) - were calculated by adding the areas under the peaks (obtained from the integrator of the gas chromatograph) for the six acids and dividing the area under each

peak by the total. By the second day after NDFDA injection, stearic, arachidonic, and docosahexaenoic acid fractions were reduced, and oleic and linoleic fractions were increased (Figure 10). At day 2 after PFOA exposure, palmitic and oleic acid fractions were increased and stearic and arachidonic acid decreased. By day 4, changes with PFOA were resolving, but those with NDFDA were increasing (Figure 11). On the eighth day (Figure 12), PFOA-treated rats exhibited a higher than normal fraction of oleic acid but this was decreasing relative to its value on day 4. On both days 8 and 16, NDFDA-exposed animals exhibited markedly increased fractions of palmitic and oleic acids and decreased fractions of stearic and arachidonic acids. By day 16 (Figure 13), the changes noted with NDFDA were slightly less than at day 8. Only NDFDA rats were evaluated at day 30. At this time, palmitate, stearate, arachidonate, and docosahexaenoate values were near control levels but oleate was still increased and linoleate was decreased.

Another method of analyzing these data is to compare ratios of specific fatty acids which have an established relationship. Stearate is primarily formed from palmitate by chain elongation. Oleic acid is formed from stearic acid by desaturation. Arachidonic acid is believed to be formed from linoleic acid by elongation and desaturation. Because of these interrelationships, it is of interest to evaluate the stearate:palmitate (SPR), oleate:stearate (OSR), and arachidonate:linoleate ratios (ALR). At day 2, the SPR and ALR were decreased and the OSR increased for NDFDA-treated animals. The SPR was also decreased and OSR increased in PFOA-treated animals (Table 2). At day 4, the same conditions existed. At day 8, the SPR and ALR were decreased only for the NDFDA-treated animals while the OSR was increased for both treatment groups. By day 16, PFOA values were not significantly different from control values but SPR and ALR remained low and OSR high for animals treated with NDFDA.

Fatty acids are important constituents of biological membranes. Esterified to glycerol phosphate and cholesterol, they play vital structural roles in phospholipid and cholesterol esters within the membrane. The physical properties of membranes, in turn, are related to the fatty acid composition of these structural components. The shifts in oleic:stearic ratio and the palmitic:stearic ratio are indicative of a more fluid membrane. One postulate proposed to explain NDFDA toxicity is that it causes a metabolic defect in fatty acid metabolism, eventually causing synthesis of abnormal or non-functional membranes within the organism. We have attempted to determine if membranes throughout the rat might be altered during the course of NDFDA toxicity. To do this, we determined red blood cell fragility at various times after NDFDA injection (Figure 14). Cell volume of erythrocytes from treated rats was increased and the cells became more resistant than control cells to hypo-osmotic conditions. In addition, at these same times, the fluidity characteristics of these cell membranes were estimated by examining fluorescent depolarization

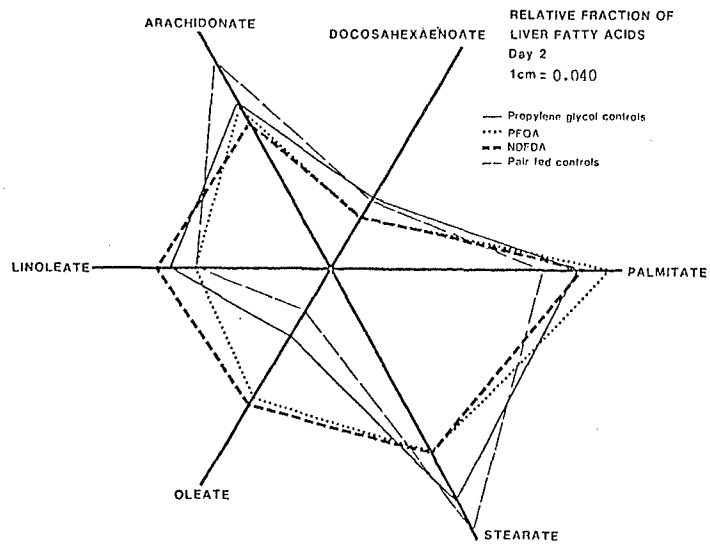

Figure 10. The relative fractions of the six major fatty acids in the livers of rats two days after treatment with 50 mg NDFDA/kg, 100 mg PFOA/kg, 2 ml propylene glycol-water (1:1)/kg or propylene glycol-water with pair feeding. Each equally separated "spoke" represents one fatty acid with the center representing 0.0 and each centimeter from the center equaling a relative fraction of 0.040. The relative fractions for each treatment are connected to illustrate differences and similarities in response.

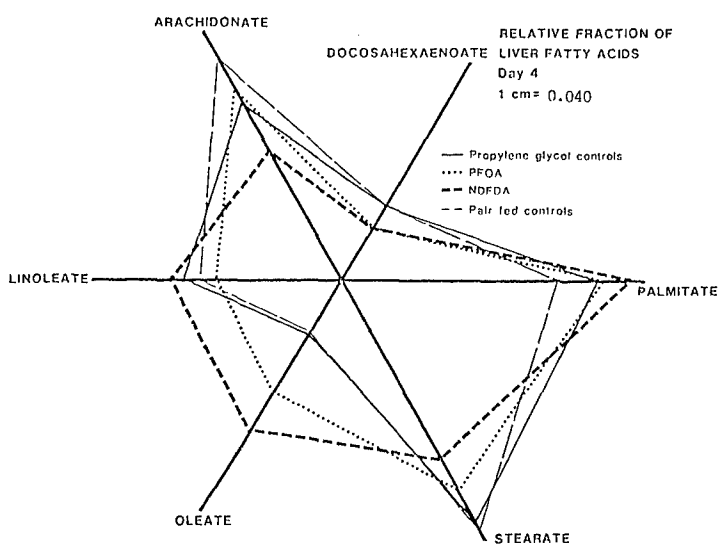

Figure 11. The relative fractions of the six major fatty acids in the livers of rats four days after treatment with 50 mg NDFDA/kg, 100 mg PFOA/kg, 2 ml propylene glycol-water (1:1)/kg or propylene glycol-water with pair feeding. Each equally separated "spoke" represents one fatty acid with the center representing 0.0 and each centimeter from the center equaling a relative fraction of 0.040. The relative fractions for each treatment are connected to illustrate differences and similarities in response.

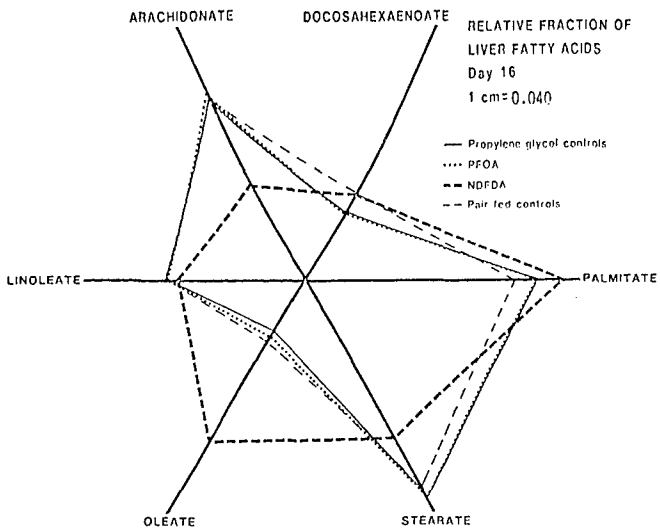

Figure 12. The relative fractions of the six major fatty acids in the livers of rats eight days after treatment with 50 mg NDFDA/ kg, 100 mg PFOA/ kg, 2 ml propylene glycol- water (1:1)/kg or propylene glycol-water with pair feeding. Each equally separated "spoke" represents one fatty acid with the center representing 0.0 and each centimeter from the center equaling a relative fraction of 0.040. The relative fractions for each treatment are connected to illustrate differences and similarities in response.

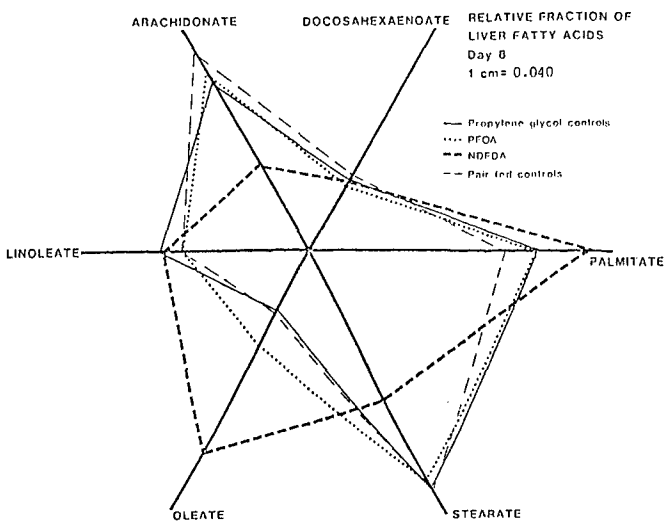

Figure 13. The relative fractions of the six major fatty acids in the livers of rats sixteen days after treatment with 50 mg NDFDA/ kg, 100 PFOA/kg, 2 ml propylene glycol-water (1:1)/kg or propylene glycol-water with pair feeding. Each equally separated "spoke" represents one fatty acid with the center representing 0.0 and each centimeter from the center equaling a relative fraction of 0.040. The relative fractions for each treatment are connected to illustrate differences and similarities in response.

TABLE 2. LIVER FATTY ACID RATIOS

TABLE 2. LIVER FATTY ACID RATIOS.

		Propylene Glycol	PFOA	NDFDA	Pair-Fed
Day 2	SPR[a]	1.061 ± 0.046[d]	0.755 ± 0.022[f]	0.831 ± 0.035[f]	1.400 ± 0.033
	OSR	0.305 ± 0.057	0.716 ± 0.055[e]	0.753 ± 0.050[e]	0.165 ± 0.010
	ALR[c]	1.184 ± 0.053	1.378 ± 0.048	0.965 ± 0.058[f]	1.779 ± 0.072
Day 4	SPR	1.101 ± 0.040	0.938 ± 0.030[f]	0.732 ± 0.046[f]	1.329 ± 0.050
	OSR	0.224 ± 0.010	0.536 ± 0.026[e]	0.844 ± 0.024[e]	1.329 ± 0.027
	ALR	1.300 ± 0.020	1.816 ± 0.098	0.925 ± 0.125[f]	1.852 ± 0.051
Day 8	SPR	1.087 ± 0.031	1.055 ± 0.043	0.542 ± 0.009[f]	1.279 ± 0.018
	OSR	0.239 ± 0.017	0.405 ± 0.057[e]	1.371 ± 0.070[e]	0.266 ± 0.026
	ALR	1.312 ± 0.030	1.648 ± 0.068	0.687 ± 0.041[f]	1.833 ± 0.059
Day 16	SPR	1.090 ± 0.043	1.082 ± 0.034	0.698 ± 0.026[f]	1.128 ± 0.035
	OSR	0.244 ± 0.011	0.268 ± 0.014	1.050 ± 0.093[e]	0.322 ± 0.043
	ALR	1.380 ± 0.033	1.461 ± 0.029	0.867 ± 0.053[f]	1.404 ± 0.112

[a] Stearate to palmitate ratio
[b] Oleate to stearate ratio.
[c] Arachidonate to linoleate ratio.
[d] Mean for 6 rats ± standard error.
[e] Significantly greater than control values at 0.05 level.
[f] Significantly less than control values at 0.05 level.

of the lipophilic dye, diphenylhexatriene. The depolarization of the dye in red blood cell ghosts was decreased (Figure 15). These data suggest the membrane has become more distensible and presumably more fluid. This would be expected if the fatty acid changes observed in liver were also reflected in the red blood cell membrane.

Another very different line of investigation has also yielded results that are indicative of altered surface chemistry of cells treated with NDFDA. These studies concerned the toxicity of NDFDA in vitro in a L5178Y mouse lymphoma cell line. These cells, used extensively in mutation research, will grow both in suspension and in semisoft agar. Below concentrations of NDFDA which cause cell lysis from its surfactant action (>150 - 200 μg/mℓ), NDFDA did not affect growth in suspension (Figure 16). After cells in suspension were treated for 24 hours with concentrations between 1 and 100 μg/mℓ, however, they would not form clones when plated in semisoft agar. Thus, cells would not proliferate when constrained to grow in close proximity to each other. The impairment of clone-forming ability was reversible after treated cells were grown in fresh suspension for 36 hours. The structure activity relationship for the impairment of clone-forming ability was striking (Table 3). PFOA did not impair clone-forming ability at any concentration where it was non-toxic in suspension. Straight chain hydrogenated fatty acids were also unable to dissociate suspension toxicity from toxicity in the semisoft agar. All tested

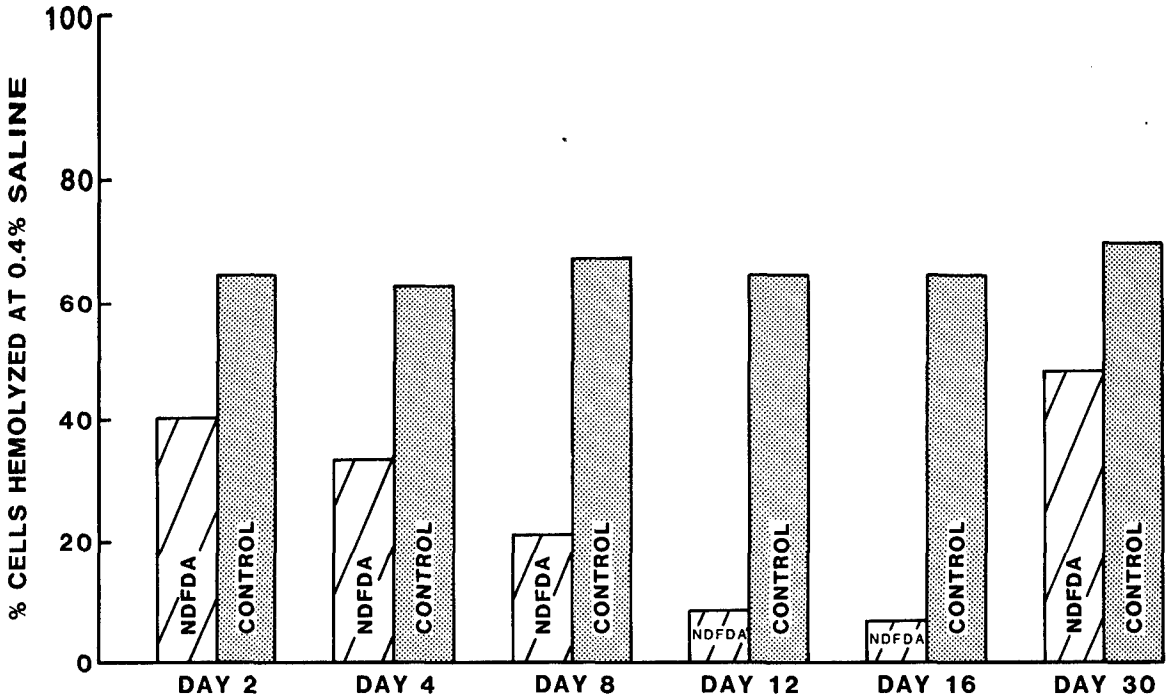

Figure 14. Hemolysis of rat erythrocytes in 0.4% saline after treatment of animals with 50 mg NDFDA/kg IP.

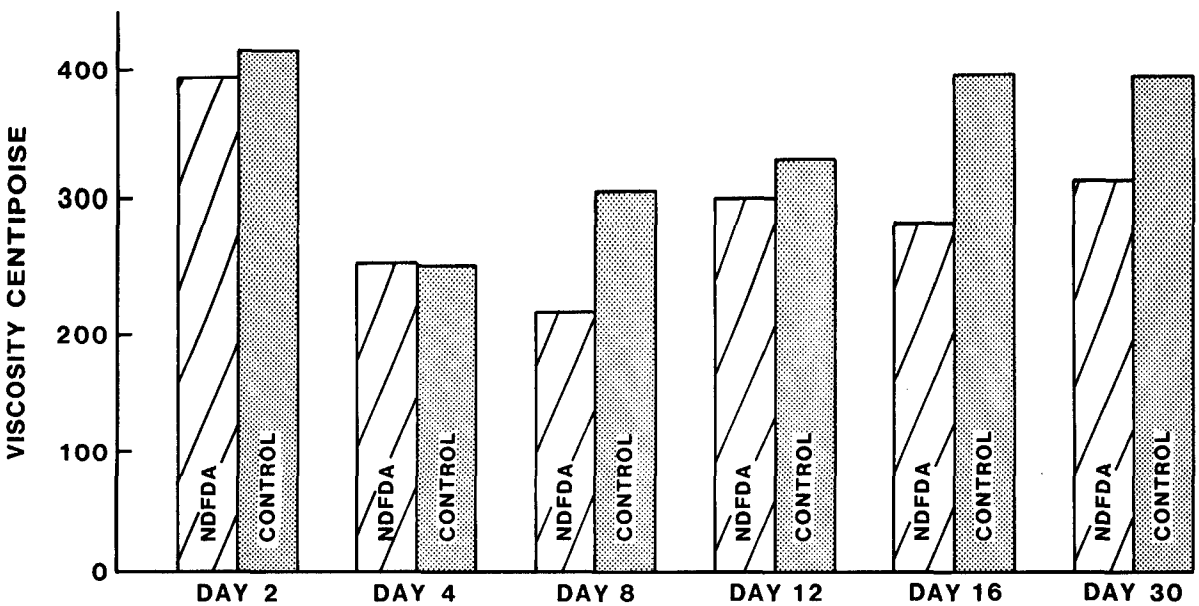

Figure 15. Viscosity of rat erythrocyte cell membranes as measured by diphenylhexatriene depolarization after treating animals with 50 mg NDFDA/kg IP.

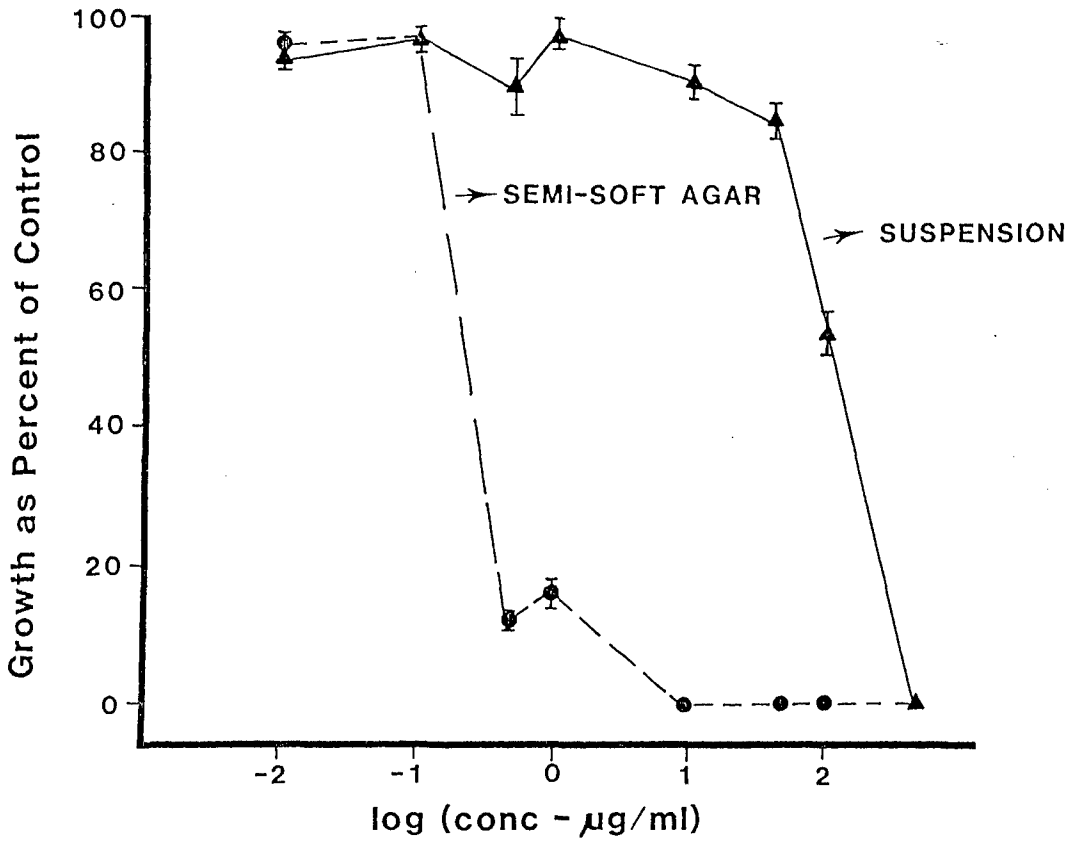

Figure 16. Growth of L5178Y mouse lymphoma cells after exposure to NDFDA for 24 hours.

perfluorinated acids of chain length 10 or greater caused impaired clone-forming ability. The ω-hydro C9 perfluorinated acid was intermediate in efficacy between the C8 and C10 acids. These findings illustrate an exquisite structure activity relationship for perfluorinated acid toxicity in these cells and bolster the conclusion that the differences in PFOA and NDFDA toxicity in vivo are truly qualitative differences. An alternative explanation that PFOA is more rapidly eliminated and that differences observed are quantitative seems less likely in view of the results in the L5178Y cell line.

This presentation has summarized the results of several research projects in our laboratory at Wright-Patterson Air Force Base. These studies were designed to evaluate the sequelae of NDFDA intoxication in rodents and eventually to give insight into the mechanism of toxicity of this acid and similarly-acting compounds. Our data are suggestive that there are alterations in fatty acid metabolism caused by NDFDA treatment and subsequent alterations in membrane structure and function in vivo and in vitro. Some interference with physiologic control mechanisms regulating fatty acid synthesis/utilization may be responsible for the toxicity of this material. The changes in fatty acid

TABLE 3. THE EFFECTS OF VARIOUS POLYFLUORINATED FATTY ACIDS ON GROWTH OF L5178Y MOUSE LYMPHOMA CELLS IN SUSPENSION AND ON THEIR CLONE-FORMING ABILITY IN SEMI-SOFT AGAR

TABLE 3. THE EFFECTS OF VARIOUS POLYFLUORINATED FATTY ACIDS ON GROWTH OF L5178Y MOUSE LYMPHOMA CELLS IN SUSPENSION AND ON THEIR CLONE-FORMING ABILITY IN SEMI-SOFT AGAR

	Perfluoro-N-Octanoic Acid		ω-H Hexadecafluoro-N-Nonanoic Acid		Perfluoro-N-Decanoic Acid		ω-H Eicosafluoro-N-Dodecanoic Acid		Perfluoro-N-Dodecanoic Acid	
	Suspension	Agar	Suspension	Agar	Suspension	Agar	Suspension	Agar	Suspension	Agar
Dose	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth	Growth
(μg/ml)	(% Control)		(% Control)		(% Control)		(% Control)		(% Control)	
1	89[a]	98	94	95	97 ± 1.1[b]	16 ± 1.9	75	11	91	25
10	89	98	94	89	90 ± 2.2[b]	0	82	0	72	0
50	76	90	92	89	84 ± 2.3[b]	0	82	0	75	0
100	76	90	83	0	53 ± 3.6[b]	0	82	0	72	0
500	21	21	-[c]	--	-[c]	--	-[c]	--	-[c]	--

[a] Numbers in each column are growth as percent of growth of control cells.

[b] Mean ± standard error (N=3).

[c] These concentrations dissolved cells in suspension.

composition in the livers - with increasing oleic and palmitic and decreasing stearic and arachidonic acids - are not observed in pair-fed controls. In addition, we observed a dramatic restriction of food intake, decrease in body weight, and depletion of body adipose stores, yet liver weight increases sharply compared to pair-fed controls, and in hamsters there is accumulation of fat in the liver.

As Fox (1981) suggested, increased oxidation of fatty acids may lead to production of prostaglandins and other biologically active derivatives, such as leukotrienes, which may adversely affect physiologic activities. The decrease in arachidonic acid, a prostaglandin and leukotriene precursor, is consistent with this idea. It may also be that NDFDA has some direct effect on membrane function. Changes in saturation of fatty acids in cell membrane phospholipids affect both the fluidity and biochemical properties of the membrane. Work with erythrocytes and mouse lymphoma cells is consistent with a change in cell membrane function. Much work remains to establish the mechanisms initiating the various physiologic effects of NDFDA. It is noteworthy, however, that survival time at the LD_{50}, toxic signs, and histopathology found with NDFDA intoxication are strikingly similar to the effects noted after 2,3,7,8-tetrachlorodibenzodioxin (TCDD) exposure (Andersen, 1981a; ibid, 1981b). There are, however, very notable differences between the toxicities of these two polyhalogenated chemicals. Most prominent is the difference in LD_{50} of nearly three orders of magnitude in the rat. Other dissimilarities include the lack of striking species sensitivity with NDFDA, the marked testicular toxicity of NDFDA in rats, and the peroxisome proliferation induced by NDFDA in these rodent species. A working hypothesis of the possible connection between NDFDA and TCDD toxicity is depicted in Figure 17.

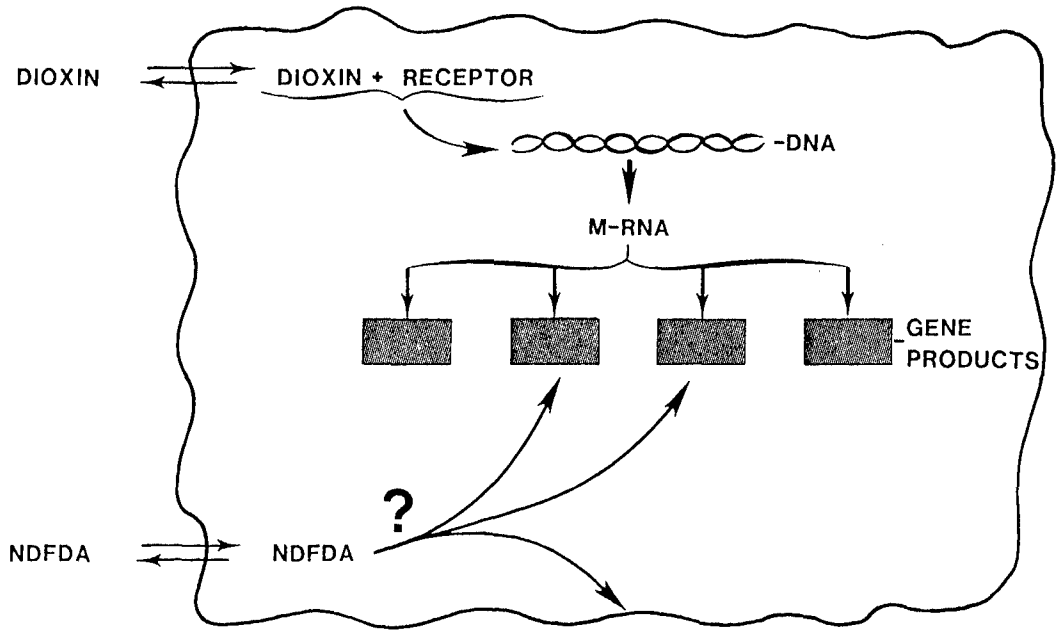

Figure 17. Hypothetical relationship of TCDD and NDFDA toxicities.

We do not imply that NDFDA and TCDD have identical mechanisms of toxicity. Instead, we believe that the perfluorinated acid affects either important gene productions (enzymes or structural components) whose synthesis is affected by the TCDD-receptor complex, or cellular constituents (e.g., membranes) whose composition is controlled by these gene products. Verification of this hypothesis requires more experimental work with both TCDD and NDFDA. At the very least, research with these toxic perfluorinated fatty acids should yield valuable leads for deciphering mechanisms of toxicity of TCDD and related compounds.

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DR. SMITH (University of Cincinnati Medical Center): Col. Olson, I noticed that you administered the compounds you studied intraperitoneally. I don't know how irritating they are, but I wondered if you had considered giving them orally in the form of a fat. How would that have affected the results? Would that have anything to do with the kinds of toxicity problems that you were concerned with? It is very interesting to me to look at these very unusual fluorinated fats which can't be dehalogenated easily. They would not be metabolized. I wondered what else you might have thought about doing with it.

LT. COL. OLSON (Air Force Aerospace Medical Research Laboratory): You might better ask that of Dr. Andersen who did some oral toxicity tests early in our program.

DR. ANDERSEN (Air Force Aerospace Medical Research Laboratory): We never tried to make an ester out of the material. We have given the material orally as the free acid or dissolved in propylene glycol and water. Its toxicity by the oral route did not vary substantially from that measured by intraperitoneal injection. We have made an ester out of that material but it was an ester with ethanol. The ester was prepared for other experiments we did to see if we got the same degree of toxicity, and we did.

DR. SMITH: How are these compounds distributed in the body?

DR. ANDERSEN: We don't know anything about its in vivo distribution at the moment. This is one of the things that we would very much like to learn but we don't know yet how it's distributed and to date we have not determined the metabolites, if any. It's unlikely that it is broken down but whether its chain is elongated and then incorporated in some other form we have no idea.

DR. CROCKER (University of California, Irvine): Dr. Peterson, I was interested in the weight loss, or rather the food spilling, you reported which sounds like a behavioral problem that makes you wonder what other behavioral abnormalities may be going on. Do you have any further input on that point or on behavioral alterations that would lead to further interest in the central nervous system?

DR. PETERSON (University of Wisconsin): We're not aware of any work that has been done in animals with respect to effects of TCDD on behavior. The food spillage was something that we happened to

observe during the course of these studies and we thought it was important enough to report. Up until this point in time, there hasn't been a great deal of effort to look at the central nervous system as a potential site of action of dioxin or other halogenated aromatic hydrocarbons. Perhaps some of the results that we have shown with respect to food intake will create interest in this area and foster research on that subject.

DR. CROCKER: Have you yourself observed any other actions that would be indications of incoordination or decreased mobility of the animal? Have you seen any behavioral effects?

DR. PETERSON: No, I haven't. I might add that when the motor movement of these animals is assessed in a treadmill test or a roto rod test, there doesn't appear to be any decrement.

DR. WHITTENBERGER (University of California, Irvine Southern Occupational Health Center): Dr. Peterson, I thought your hypothesis about a change in body weight set point was very ingeniously developed and I wondered if you know of other examples of toxic chemicals that might produce a similar effect.

DR. PETERSON (University of Wisconsin): This hypothesis of a change in body weight set point is a hypothesis that has been used to explain the loss of body weight and the reduced food intake in rats that have lesions of the lateral hypothalamus. Dr. Richard Keesey, who collaborated with us on this study, has been working in this area for a number of years. I should add that we do not have any evidence that TCDD treatment causes a lesion of the lateral hypothalamus (LH). Our original rationale for thinking that the TCDD treated animal would be similar to the LH lesioned animal is that perhaps TCDD was acting at the level of the hypothalamus to produce alteration in gene expression that would result in the same type of behavioral manifestation as in the LH lesioned animal. Dr. Gasiewicz tells me that he has looked at the hypothalamus, but I'm not sure if this was in rats for the presence of the TCDD receptor which is thought to be responsible for initiating the various responses to dioxin. He might wish to comment on what he has found.

DR. GASIEWICZ (University of Rochester School of Medicine): Under the conditions of this particular assay system, we did not find a receptor in the hypothalamus but I would caution that that only means we could not detect it by this assay.

DR. SMITH (University of Cincinnati Medical Center): I've never seen the data, but I understand that you can force-feed the

TCDD treated animals and they still die the same way. Can someone tell me how that is related to this weight loss problem that Dr. Peterson reported?

DR. GASIEWICZ (University of Rochester School of Medicine): I should respond to that question because we did those studies. The problem is complicated to begin with. We did those studies because of the weight loss problem and we wanted to answer the question of whether it was decreased food consumption or decreased utilization of nutrients. We decided to bypass the gastrointestinal system and feed the rats intravenously using total parenteral nutrition. When we did this study we gave TCDD to rats by total parenteral nutrition and they gained weight just like the normal animals on total parenteral nutrition. Under that particular treatment there was no loss of body weight which suggests that once nutrients are given to the animals they can utilize them effectively. The problem we ran into with that study was that the animals died anyhow, even though they were gaining weight. The study was also complicated by the finding that in the parenterally fed animals treated with TCDD the hepatic damage was much greater than seen in the case where the TCDD treated animals were fed ad libitum. Indeed, in the animals fed by total parenteral nutrition, it looked like the animals were dying from hepatic damage which was somehow potentiated by those feeding conditions. We are about to repeat some of those studies in the guinea pig, primarily because under ad libitum conditions the guinea pig does not appear to get hepatic damage. Those studies are not yet completed so I can't comment on them.

DR. PETERSON (University of Wisconsin): To follow up on what Dr. Gasiewicz said about the findings in TCDD treated rats that were maintained on total parenteral nutrition, their results with respect to body weight are entirely in accord with the hypothesis that was proposed today - the hypothesis being that TCDD treatment lowers the regulated level of body weight in the rat. In the experiments that were done by Dr. Gasiewicz and Dr. Neal, if you lower the regulated level of body weight in the rat but continue to feed that rat at the same rate as a control rat, that is, regulate its body weight at a higher level, you would expect to see the TCDD treated rat put on those calories inappropriately as body weight in terms of increased carcass fat. The results obtained showed that that was the case; the TCDD treated rats maintained on total parenteral nutrition weighed the same as control rats but had increased levels of carcass fat as shown by increase in visceral adipose tissue. This is the same thing that we found in TCDD treated rats when fed a high calorie diet. The rats put on weight, but they put it on as excess fat so these findings go hand-in-hand with some of the results that we showed with high calorie challenges.

DR. GASIEWICZ: Dr. Peterson, you mentioned the point about the increase in adipose tissue stores in the TPN fed animals. It's interesting that in the ad libitum fed rats there was hypoglycemia following TCDD treatment. Despite the fact that they were continuously fed a diet which contained 70 or 75 percent of the caloric intake as glucose, the animals were still severely hypoglycemic. And it looks like the calories from glucose are being put into adipose tissue so there is alteration in glucose metabolism.

DR. WHITTENBERGER (University of California, Irvine Southern Occupational Health Center): Before we leave this subject, I'd like to ask Dr. Peterson a further question. I assume that the body burden of TCDD was constant during the period of your observation. I wonder if you tried to repeat your observation in another species of animal that's much longer lived, would TCDD produce similar results or not?

DR. PETERSON (University of Wisconsin): With respect to the first part of your question, the half life of TCDD in the rat has been estimated at between 21 and 28 days. When we treated rats with 15 micrograms TCDD per kilogram and followed their weight for 90 days, the TCDD should have been eliminated from the body during that period. If we held the rats for a longer time and all the TCDD was eliminated from the animal, this effect on body weight should gradually diminish and the weights begin to converge with untreated controls. I don't think we should think of it as being an irreversible effect. I find it hard to comprehend how that could occur. With respect to your second question, we haven't looked at long-term effects of TCDD on body weight in other species, but other investigators have also reported progressive and long-term depression in body weight. However, the effects on food intake were not reported.

DR. ALI (Ohio State University School of Medicine): Dr. Aust, what is the significance of the histologic lesion you showed us?

DR. AUST (Michigan State University): We're trying to see what the mechanism of promotion might be for these compounds. My colleague, Dr. Trosk, has a theory that the inhibition of metabolic cooperativity may be either a short-term in vitro assay for promotion or it might even be a mechanism of promotion. That is, if cells cannot communicate, they have lost their ability to control each other and initiated cells may develop clones and finally a tumor. The inhibition of metabolic cooperativity could occur by some biochemical mechanism on the surface of a cell or traumatically injure the cell and it could not communicate. The question was, did we get enzyme altered foci that correlated with what a pathologist would recognize as altered histopathology? They performed histopathology and histochemistry on these cells and looked for alterations without

knowing anything about the study, or about the chemicals used for exposure. The second point was to see if the promotion of enzyme altered foci in these experiments was correlated with enzyme induction because another theory of tumor promotion is that you simply induce the enzymes in the liver over a prolonged period of time. So we used enzyme induction as one assay and histopathologic change as another assay and then measured the number of enzyme altered foci in those animals. We found that the increase in enzyme altered foci correlated, not with enzyme induction but with histopathologic changes, suggesting that it's related to toxicity. With the other congener, which is not toxic, we're asking the question, is the mechanism of promotion of enyzme altered foci correlated with enzyme induction? In this case it's a different enzyme.

DR. ALI: Did you initiate all these groups with diphenylnitrosamine?

DR. AUST: Yes, all of them are the same.

DR. ALI: The nitrosamine initiation or promotion with phenobarb doesn't show any histopathologic lesion?

DR. AUST: You see proliferation of the endoplasmic reticulum with phenobarbital but not what you would call a lesion.

DR. PETERSON (University of Wisconsin): Agents which are phenobarbital type inducers have been shown to increase liver DNA synthesis after their administration. This mitogenic action may play a role in promoting ability. With respect to 3,4,5, 3',4',5' HPB, could you be a little more specific on the mechanism by which you think it might be promoting? Are you suggesting that what might be occurring here is that you are getting hepatotoxicity in normal liver cells and these pre-neoplastic cells may be resistant to that type of negative selection pressure so that they then can proliferate under that influence? Is that the postulate that you're suggesting?

DR. AUST (Michigan State University): I'd rather not be that precise! There were also animals in this study that were used to measure tumor response. I didn't show any of those data but we have measured not only enzyme altered foci but number of tumors. We have not completed the evaluation of the data but eventually hope to correlate the number of enzyme altered foci with number of tumors.

DR. GASIEWICZ (University of Rochester School of Medicine): If my memory serves me correctly, there was a study reported by Dow Chemical a few years ago in which they conducted a two year study feeding various levels of TCDD to animals and they looked at the production of tumors in these animals. Again, if my memory serves me correctly, in cases where they saw a significant increase in tumors there was also other tissue damage such as porphyria or necrosis present.

DR. AUST (Michigan State University): That's right and it probably should be mentioned that Kimbrell did 18-month PBB feeding studies and concluded that tumors were seen only at doses that produced other toxic effects.

DR. KNUTSON (University of Wisconsin): Using the model of the HRS/J haired and hairless mice, TCDD has been shown to be a promoter in the liver. The idea occurred to people in our lab that although TCDD had not been shown to be a promoter in the normal laboratory mouse skin, that was also the mouse strain that did not show the hyperplasia response to TCDD. The question then was, if the hairless mouse were initiated would TCDD promote tumors? The answer to that question is yes, it does and that promotion effect appears to be mediated by the receptor. The hairless mouse gets tumors in response to TCDD if the mouse has been initiated with either DMBA or with MNNG but the haired mouse does not.

DR. PETERSON (University of Wisconsin): In that model do you think the promotion is tied in with the mitogenic effect of TCDD as opposed to a toxic effect on epithelial tissue?

DR. KNUTSON: It's difficult to define what a toxic effect is. There are levels that we're using where you don't see the tremendous scalyness of the epidermis. Our guess is that it is the hyperplastic type response that it's related to.

DR. REESAL (University of California, Irvine Southern Occupational Health Center): I have two questions. They both relate to the effects of dioxin on the lymphoid system. The first is this. I gather that dioxins produced a weight loss in 100% of the animals and they also produced thymic atrophy in 100% of the animals. Do you think that these effects are end results? Do you think that they are related?

DR. PETERSON (University of Wisconsin): Was the question do we think that thymic atrophy and weight loss are related effects?

DR. REESAL: Yes, I gather that most of you think that they are end results of the toxicity of dioxin. I think Dr. Gasiewicz indicated they might be related. Is it the opinion of the panel that they're not related at all?

DR. GASIEWICZ (University of Rochester School of Medicine): I suggested that possibility, but I believe up to this point we just really don't know.

DR. REESAL: May I ask a second question, please? Do you have any information on the rest of the lymphoid system since this effect on the thymus is such a profound change? Do we know what happens to the lymph nodes or to the peripheral lymphocytes?

DR. GASIEWICZ (University of Rochester School of Medicine): I believe that there is some effect upon the peripheral lymphoid tissue but I believe the effect on the thymus tissue is the most profound.

DR. AUST (Michigan State University): We have not done any work with TCDD but a colleague has worked with PBB's and I'm taking the liberty of saying that 3,4,5,3',4',5' hexabromobiphenyl has the same toxicity as TCDD and the cells which have been processed by the thymus are no longer affected by the 3,4,5,3',4',5' HBP, so it gets very complicated. The toxic effect appears to involve the lymphocyte processing machinery within the thymus. There may also be effects on cell processing machinery other places. I think we could probably take many cell processing systems, bone marrow, thymus, skin, or glands and show an effect by these substances. That's my hypothesis.

DR. REESAL (University of California, Irvine Southern Occupational Health Center): I think the effects are rather interesting and it's probably unique that you are getting a central lymphoid lethal effect when it is not affecting the peripheral lymphoid tissue at all.

DR. AUST: Exactly. I think that is very interesting. I don't want to say that the only effect is on the thymus, however. There may be other tissues that are involved in post-mitotic differentiation of cells. But, of course, with T cells the effect is at the thymus it would appear.

DR. WHITTENBERGER (University of California, Irvine Southern Occupational Health Center): In the studies that Greenlee and Rice

have done with cultured human epithelium, I believe they found that the effects of TCDD were influenced by the level of cortisone. Are you familiar with that study? Does it have any possible effect upon any of the findings that you have been presenting today?

DR. KNUTSON (University of Wisconsin): The corticosteroids do not bind to the TCDD receptor and TCDD does not seem to bind to the corticosteroid receptor so if there is an interaction between those two it's someplace beyond that for both of those compounds.

DR. PETERSON (University of Wisconsin): In the intact animal there doesn't appear to be adrenal hyperfunction and in that regard you can remove the adrenal gland from these animals and you still see delayed lethality. It doesn't appear to be related to an increase in circulating levels of corticosterone.

COL. DALTON (U.S. Army Environmental Hygiene Agency): As a medical officer engaged in environmental and occupational health, I was fascinated by Dr. Peterson's hypothesis because we frequently deal with subjective complaints of anorexia and weight loss from all exposures to dioxin. First of all, I would like some clarification on a point. I think you said that all animals in your study lost weight transiently but only in those that received greater than 25 micrograms per kilogram did this weight loss persist according to your hypothesis. Is that correct?

DR. PETERSON (University of Wisconsin): The one thing that I think we should say is that we had given doses of 5, 15, 25, and 50 micrograms per kilogram to groups of animals and the animals dosed with 25 and 50 µg/kg lost weight and lethality occurred at a body weight that was approximately 50% of the ad libitum control groups. There was a loss in body weight that persisted for 35 days. We have then observed animals dosed at the 15 µg/kg level for 90 days and recently for 110 days and these animals maintained body weights roughly 15% less than that of control groups but it appears that at 110 days the weight is slowly beginning to come back and instead of being 15% lower now it's 14%-13%. I think eventually these effects will be reversed. I don't believe it is a permanent effect.

COL. DALTON: Is it safe then to extend your hypothesis to the very low dose levels that this may represent an alteration in the set point for weights which is reversible?

DR. PETERSON: That's a difficult question to answer. We have to keep in mind this is just a hypothesis and we need to do a lot more experimentation with it. I'm very hesitant to take what we

found in the rat and to start applying it to the human situation when we haven't even applied it to another animal species yet. I think we need to exercise a great deal of caution and keep in mind that the purpose of this hypothesis was to stimulate new ideas for doing research on TCDD.

COL. DALTON: Thank you. I accept your caution and certainly will heed it. Unfortunately, we as users must take the rat data and apply it to humans all the time so this is a fascinating hypothesis and does stimulate me to go back and look at our data just to see what the true weight loss and anorexia profiles are for dioxin exposed humans.

DR. PETERSON (University of Wisconsin): I would like to ask a question of the panel. I think some of the work that has been done by the Air Force with these perfluorinated fatty acids, PFDA, is fascinating. I don't think it was an accident that this research was presented along with the work that's been done on halogenated aromatic hydrocarbons and I think the panel should address a question about it. Do you think that the toxicity that's produced by these agents is by a similar mechanism to the halogenated aromatic hydrocarbons and if so, why and if not, why not?

DR. AUST (Michigan State University): I think that's a very good comment and I would like to point out something to Drs. Olson and Andersen. When vitamin A deficient animals are given PBB, bile duct proliferation is one of the pathologic changes produced. We could have given an entire paper on bile duct proliferation in animals given PBB with a concurrent vitamin A deficient diet. I was really struck by the similarities there. But in general I think one of the first things to do would be to conduct receptor binding assays to see if the perfluoro compounds are causing more of these striking similarities.

DR. PETERSON (University of Wisconsin): Dr. Andersen, what is your hypothesis with respect to the mechanism of action of the perfluoro compounds you studied? To what extent do you think it might be similar to other halogenated aromatic chemicals and if it's different, where is it different? What is your working hypothesis?

DR. ANDERSEN (Air Force Aerospace Medical Research Laboratory): What we should do is get the four or five people who have contributed to this work to come up and give you the hypothesis that they favor at the moment. But I'll start. The idea that we have is that there is a similarity in toxicity in the phenomenology. But that doesn't necessarily mean there is a detailed equivalency of mechanism. My working hypothesis goes something like this. Dioxin has a

receptor, it affects control at the level of DNA with repression or induction of messenger RNA and then controls a certain number of gene products: proteins, enzymes, materials involved in the different metabolic pathways, materials that would also be hormonally controlled by vitamin A, by thyroxin, or by some other endogenous hormonal control system. I think that we just stumbled upon a chemical, perfluorodecanoic acid, that has some effect on preformed constituents of the cells - perhaps those gene products whose production is being controlled by TCDD and its receptor. I'd be very much surprised if the perfluorinated decanoic acid were able to bind to your receptor. It would be disappointing to me if it did. This is in general the working hypothesis. That doesn't tell much about the mechanism of toxicity and how the thymus is affected. Several times in the discussion today there have been aspects on the thymus, on the testes, on the changes in the liver of animals fed by total parenteral nutrition. I think our work has something to contribute but I'm very reluctant to discuss it now with you because it's incomplete. We are talking about two chemicals whose biologic effects are the same but whose mechanisms are probably very different. I would point out to the audience that the constellation of toxic signs which have been seen with dioxin and with certain polybrominated and polychlorinated biphenyls were considered unique to those chemicals. We were really quite surprised to find these effects with perfluorodecanoic acid. And although it wasn't remarkable, it's even more surprising, in a way, that we don't see it with perfluorooctanoic acid because there is a very striking structure activity relationship in the toxicity to the animals and also in the toxicity to the cells that we were looking at.

LT. COL. OLSON (Air Force Aerospace Medical Research Laboratory): Dr. Lubet, earlier this afternoon, was talking about some initial studies that he did in which he found that there was no AHH induction with NDFDA, so presumably it does not operate the same way. However, it seems to produce the same signs and same effects as these other halogenated materials. I have no single hypothesis to offer.

DR. PETERSON (University of Wisconsin): The one thing that I had noticed from Dr. Olson's presentation was early lethality. With TCDD you cannot get lethality in less than say 10 to 14 days at the earliest in many species. However, was I correct in interpreting your slide that by increasing the dose of PFDA can get lethality occurring within a day after treatment?

LT. COL. OLSON: It's not usually right away unless a dose is very high. If the dose is high enough it will kill within a few hours. With PFOA, if the animals are going to die they'll usually die very early and with NDFDA, increasing dose decreases survival time within a few days.

DR. PETERSON: The question is whether this should detract from the similarity of these compounds or not? I heard Dr. Aust mention that he thought perhaps the early death was a different mechanism than that involved with the delayed onset death that you get with lower doses, is this right? Does this fit with your line of thinking?

LT. COL. OLSON: I would tend to believe the same thing, yes. The dose response is a biphasic curve where you have early deaths at high doses with one mechanism and then later on a second mechanism appears to be involved.

DR. PETERSON (University of Wisconsin): Dr. Aust, does reduced food intake affect vitamin A levels in rats? When you feed your rats at 100 parts per million PBB, do they lose body weight?

DR. AUST (Michigan State University): The answer to the first question is extremely complex. You just cannot start altering vitamin A levels by fooling around with body weight or feed intake. The animal's body regulates its vitamin A very carefully. Probably because it's toxic. If you decrease food intake and then start worrying about now what was their intake of vitamin A, it doesn't make any difference. You can do anything you want to a normal diet and the animal absorbs a certain amount and it does this in trying to maintain a constant level of a certain form of vitamin A in the liver. It can do this by absorbing another form and going through certain biochemical changes. It gets very complicated. When we ran onto this observation we went straight over to an expert and said tell us everything you know about vitamin A metabolism and its role and she said I have very sad news for you, we don't know much! We do know how it is controlled so that you can't manipulate it. In other words, it was bad news experimentally. Secondly, the body weight changes that are seen with TCDD are also seen with PBB. The response is almost exactly the same and it looks to me like it's the same mechanism. The interesting thing is that if you decrease body weight down to a certain level it would appear that's when the animal is basically destined to die. If you don't decrease it quite that much by decreasing the dose a little bit, it'll recover and it eventually goes back to the control level. It may be that there is a difference here between TCDD and PBB because one is metabolized and the other is not. Since the PBB is there forever, we can't do the experiment, but interestingly enough, that animal is trying to maintain its liver storage form of vitamin A and when it can't do that the animal is destined to die. You can't then give them an injection or increase the intake of vitamin A as we get decreased food intake. It doesn't work.

DR. PETERSON: Can you do pair feeding studies in these rats?

DR. AUST: Yes.

DR. PETERSON: Can you then find out if the alterations in serum liver vitamin A levels are the same or different in the PBB and the pair fed control groups? They should both be getting the same intake of vitamin A. I think that would be an important control to find out if these effects on vitamin A are secondary to the reduced food intake that you see in these animals.

DR. AUST: You can easily pair feed them, but by that time you cannot change vitamin A levels enough by starvation, excess feeding or anything else unless you put the animals on a special diet before you ever start your treatment with the toxin. It's very difficult to do anything even if their intake is different. You can do pair feeding and basically you affect one vitamin A form, retinal palmitate in serum, but that's not the critical form. It's retinone in the liver which can't be influenced during 15 days much anyway, no matter what you do to the animal.

LCDR WYMAN (Naval Medical Research Institute): I wanted to address my questions to either Dr. Olson or Dr. Andersen. Dr. Aust pointed out the similarity between the symptoms of vitamin A deficiency and the toxicity of TCDD and you have a compound that exhibits a similar toxicity to TCDD. Is there a relationship of PFDA to vitamin A? Perhaps a structural analog relationship?

LT. COL. OLSON (Air Force Aerospace Medical Research Laboratory): We did not see a relationship because while we have several strains we have not used different strains of the same species. We didn't see any hyperkeratosis which would be a sign of the vitamin A deficiency. Dr. Andersen, do you want to talk about the rabbit?

DR. ANDERSEN (Air Force Aerospace Medical Research Laboratory): Yes. There are a number of similarities between the toxicity of perfluorodecanoic acid and dioxin and we've gone over some of them. There are some dissimilarities and some of them were mentioned. The testes are much more dramatically affected in the animals treated with PFDA and the animals actually stop eating. They don't just restrict food intake. They stop eating. We didn't discuss all the experiments we've done but another difference is that we've been unable to induce the skin lesions in a rabbit ear with perfluorodecanoic acid. And I don't know if that's just a failure of our technique but we attempted the experiment and we've not been able to get hyperkeratinosis.

SESSION V

HYDROCARBON TOXICOLOGY

Chairman

LCDR Anthony P. D'Addario, MSC, USN
Naval Medical Research Institute
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PAPER NO. 21

INHALATION EXPOSURE STUDY OF A SOLVENT REFINED COAL MATERIAL

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INTRODUCTION

Batelle-Northwest is engaged in evaluation* of the toxicologic effects of solvent-refined coal (SRC) materials. Our approach has been to develop a broad base of toxicologic data built on a rigorous chemical characterization of individual components. Initially, several SRC materials were evaluated for mutagenic and mammalian cell transformation activity (Pelroy and Stewart, 1981). Materials that had biological activity in the in vitro assays were evaluated in whole-animal studies such as chronic skin painting in mice (Renne et al., 1981) and the initiation promotion assay in mouse skin (Mahlum, 1982). In addition, other studies such as assays for teratogenic events (Springer et al., 1982) and dominant lethality were conducted. Since the high-boiling materials were active as skin carcinogens, we have extended these studies to include an inhalation exposure study. The material selected for this study was heavy distillate (HD), the 550° - 850°F fraction from the SRC-II process, obtained from the pilot plant at Ft. Lewis, WA, which is operated by the Pittsburg & Midway Coal Mining Co.

METHODS

The exposure chamber selected for use in these studies was developed in our laboratory (Moss et al., 1982). For our studies, the chambers were operated at 283 L/min with air movement from top to bottom. Aerosol was produced by a Solo-sphere nebulizer operated at 35 lbs per sq. in. The aerosol first moved into a 5-L aging chamber and then into a manifold system before delivery to the chambers. Aerosol within the stainless steel manifold, which was operated at a slightly negative pressure relative to the room, was drawn into the chambers through an orifice-controlled delivery

*Work supported by the U.S. Department of Energy under Contract No. DE-AC06-76RLO-1830.

system (Springer et al., 1982). Samples of aerosol from the chamber were collected onto Metricel filters by withdrawing air using a 1-L syringe. Material collected on these filters was eluted into chloroform and the amount of coal liquid was determined by ultraviolet absorption at 254 nm. Prior to beginning exposure of animals, we established that the aerosol concentration and particle size were uniform throughout the exposure chamber.

For the subchronic inhalation study, animals were exposed 6 hours/day, 5 days/week, for 13 weeks. The average aerosol concentrations (± SEM) for the three treatment groups over the 13-week exposure period were 0.69 ± 0.0036, 0.14 ± 0.0015, and 0.029 ± 0.0004 mg/L; control animals were exposed to filtered room air only. The aerosol particle size in the three chambers during the exposure period ranged between 1.7 and 1.8 μm mass median aerodynamic diameter (MMAD), indicating that a large fraction of the inhaled material was likely to be deposited in the deep lung.

For this experiment, 32 male and 32 female Fischer 344 rats were exposed for each treatment group. The rats were first weighed 2 weeks prior to exposure, individually marked, and randomly assigned by weight to an exposure group.

After 30 days of exposure, 10 male and 10 female rats/treatment group were selected for evaluation. Blood samples were collected for hematology and clinical chemistry evaluation, and selected organs from these animals were removed and weighed. At the end of 13 weeks of exposure, 15 males and 15 females per group were bled and all remaining animals were sacrificed and necropsied.

RESULTS

Weekly body weight data for male and female rats are shown in Figures 1 and 2, respectively. For male rats, there was a dose-related decrease in body weight gain. The control group grew at a steady rate during the exposure period, gaining approximately 120 g. Body weight gain for animals in the low-dose group was not significantly different from that of the control group. Animals in the mid-dose group consistently gained significantly less weight than the controls ($P < 0.05$). The animals receiving the high dose lost weight during the first 2 weeks of exposure, then failed to gain weight throughout the remainder of the exposure period. Similar effects on body weight gain were observed for female rats (Figure 2), even though the absolute differences in body weight for the females were less dramatic than for the males. Effects for both males and females were clearly dose related. These data indicate that exposure to HD at 0.69 and 0.14 mg/L resulted in a significant alteration in growth for male and female rats; however, at 0.029 mg/L, very little effect was observed.

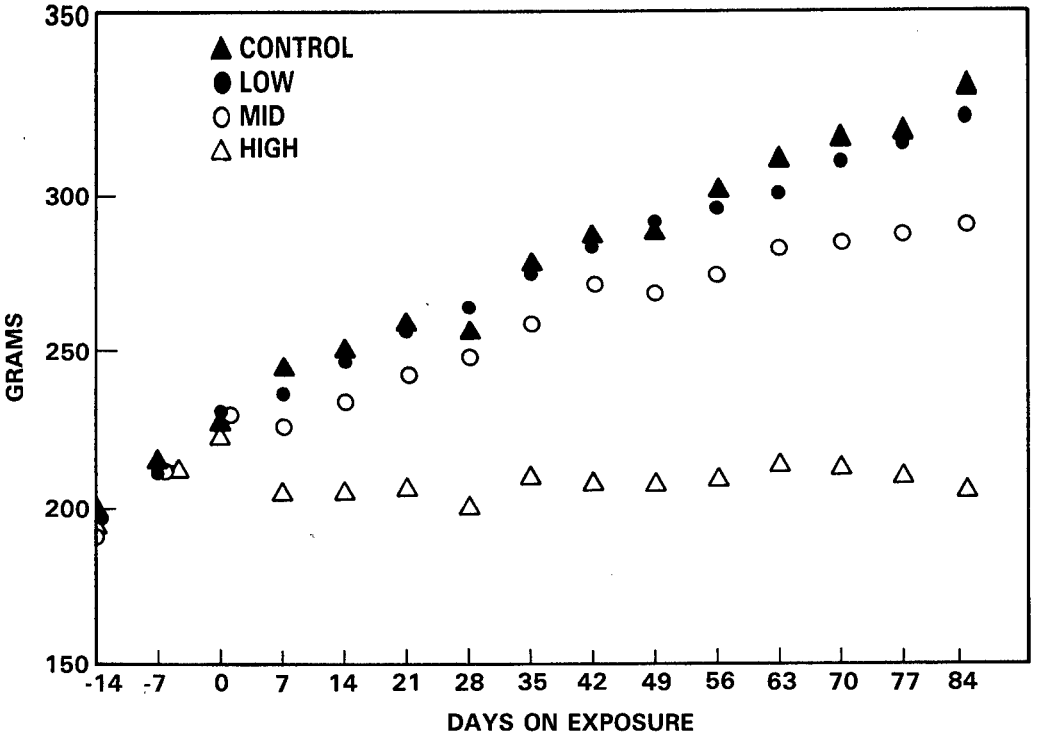

Figure 1. Body weight gain for male rats exposed to heavy distillate by inhalation.

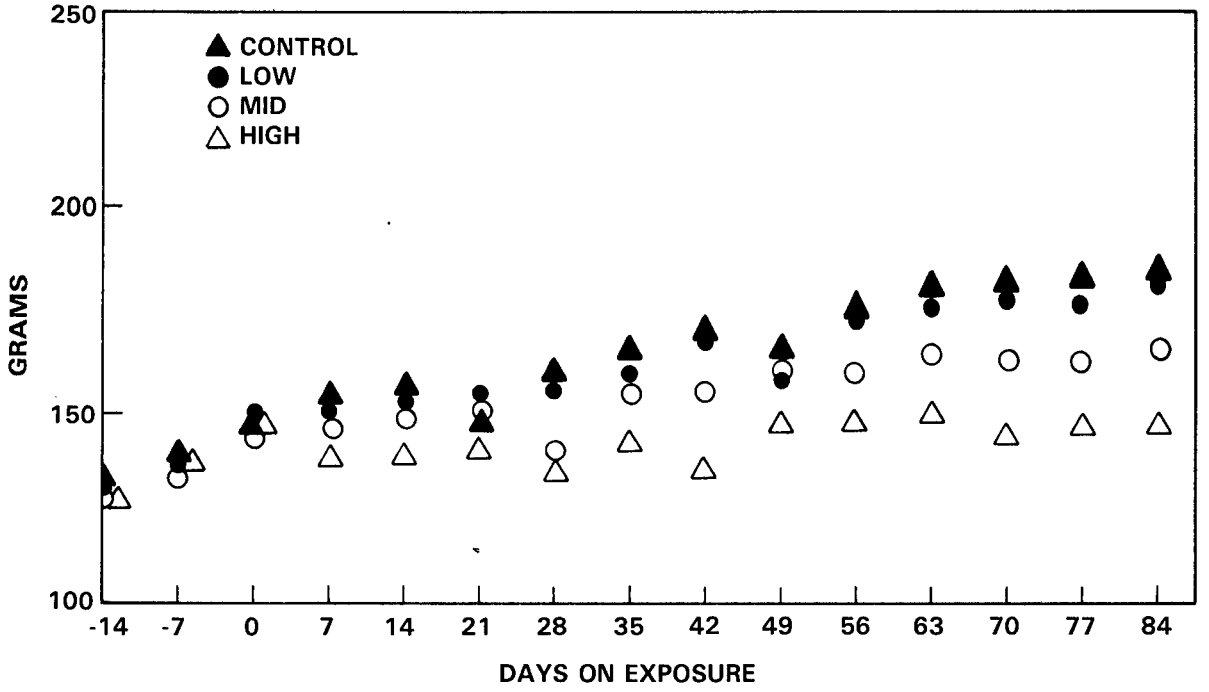

Figure 2. Body weight gain for female rats exposed to heavy distillate by inhalation.

Even though body weight gain was severely depressed for animals in the high-exposure group, 94% of these rats survived the 13 weeks of exposure (Table 1). Most deaths occurred during the last 3 weeks of the exposure.

TABLE 1. SURVIVAL OF RATS DURING 13-WEEK INHALATION EXPOSURE TO HEAVY DISTILLATE

Sex	Treatment Group	No. Animals Exposed	No. Deaths	Mortality (%)
Male	Control	32	0	---
	Low	32	0	---
	Mid	32	0	---
	High	32	2	6
Female	Control	31	0	---
	Low	32	1	3
	Mid	31	1	3
	High	31	2	6

A complete necropsy (approximately 35 tissues) was performed at the end of each exposure, including weights of heart, liver, lung, spleen, thymus, brain, kidneys, adrenals, and gonads. The data in Table 2 show tissue weight expressed as percent of body weight for female rats sacrificed after 30 and 90 days of exposure. For these animals, there was a significant increase in absolute liver weight in the low-, mid-, and high-dose groups after 30 days of exposure, with a significant trend for an increase in liver weight as the dose increased. Following 90 days of exposure, absolute liver weight increased relative to controls for animals in the mid- and high-exposure groups. On a relative tissue weight basis, significant increases were observed for all three exposure groups after 30 days of exposure and for the mid- and high-dose groups after 90 days of exposure. Liver weight for male rats increased in a similar manner; however, significant increases were consistently observed for all three dose groups.

In addition to the effects on the liver, absolute mean weights for thymus were significantly lower than those for the control group. After 30 days of exposure, thymus weight decreased dramatically for female rats in the high-exposure group

TABLE 2. TISSUE WEIGHT AS PERCENT OF BODY WEIGHT FOR FEMALE RATS AFTER EXPOSURE TO HEAVY DISTILLATE[a]

Treatment Group	Liver	Thymus	Ovaries
	30-DAY EXPOSURE		
Control	3.21 ± 0.05	0.152 ± 0.014	
Low	3.52 ± 0.05[b]	0.132 ± 0.007	
Mid	3.84 ± 0.07	0.131 ± 0.007	
High	5.49 ± 0.10[c]	0.054 ± 0.010[c]	
	90-DAY EXPOSURE		
Control	3.54 ± 0.09	0.13 ± 0.009	0.039 ± 0.002
Low	3.55 ± 0.08	0.11 ± 0.005	0.036 ± 0.001
Mid	4.18 ± 0.12	0.09 ± 0.005	0.037 ± 0.001
High	6.37 ± 0.11[c]	0.05 ± 0.006[c]	0.026 ± 0.002[c]

[a] $\bar{x}$ ± SEM.

[b] Underlined values are significantly different from controls ($p < 0.05$).

[c] Significant dose-related trend.

(Table 2). By 90 days of exposure, thymus weights for all three exposure groups were significantly less than those for controls; these decreases were dose-dependent. Thymus weights also decreased for male rats in a manner similar to those for females.

Ovaries and testes were weighed only after 90 days of treatment. Absolute ovary weights were significantly less than those of controls for animals in the mid- and high-exposure groups (Table 2). In addition, the weights of testes, heart, kidney, spleen, brain, and adrenals decreased on an absolute basis; however, on a relative weight basis, the weights of these tissues increased. Our interpretation of these data was that these organ differences did not reflect biologically significant effects that were attributable to the exposure.

TABLE 3. MEAN ERYTHROCYTE PARAMETERS FOR FEMALE RATS AFTER EXPOSURE TO HEAVY DISTILLATE

	Control	Low	Mid	High
	30-DAY EXPOSURE			
HCT (%)	40.7	39.6	37.6[a]	33.9
Hgb (g/100 ml)	16.1	15.6	14.6	13.2
RBC (10^6/mm^3)	8.1	7.8	7.5	7.2
Reticulocytes (10^3/mm^3)	166.6	175.8	261.4	422.9
Nucleated RBC (10^3/mm^3)	0.043	0.004	0.006	0.030
	90-DAY EXPOSURE			
HCT (%)	44.2	42.6	40.7	27.0[a]
Hgb (g/100 ml)	17.2	16.6	15.7	10.3
RBC (10^6/mm^3)	8.3	8.0	7.8	5.2
Reticulocytes (10^3/mm^3)	90.4	116.6	85.8	293.8
Nucleated RBC (10^3/mm^3)	0.007	0.026	0.024	0.090

[a] Underlined values are significantly different from controls ($p < 0.05$).

Blood samples obtained at 30 days were significantly decreased compared with those of controls, with regard to the volume of packed red cells (HCT), hemoglobin concentration (Hgb), and the number of red blood cells (RBCs), and increased with regard to the number of reticulocytes (Table 3). These effects were observed for female rats in the mid- and high-exposure groups. After 90 days of exposure, similar results were observed although the changes were not always significant for the mid-dose group. In addition, there was a significant increase in the number of nucleated RBCs in samples from animals in the high-dose group.

Blood samples were also evaluated for exposure-related changes in the leukocyte cell series. Following 30 days of exposure, the only significant change in these cells was a decrease in the number of eosinophils from animals in all three

TABLE 4. MEAN WHITE BLOOD CELL COUNT FOR FEMALE RATS AFTER EXPOSURE TO HEAVY DISTILLATE[a]

	Control	Low	Mid	High
	30-DAY EXPOSURE			
Total WBC	6.5	4.8	6.0	6.0
Lymphocytes	4.8	4.6	4.5	4.3
Neutrophils	1.6	1.1	1.5	1.7
Eosinophils	0.06	0.03[b]	0.03	0.01
Monocytes	0.02	0.02	0.02	0.01
	90-DAY EXPOSURE			
Total WBC	7.7	7.8	7.1	5.8[b]
Lymphocytes	6.0	6.1	5.8	4.2
Neutrophils	1.5	1.6	1.2	1.6
Eosinophils	0.09	0.04	0.03	0.002
Monocytes	0.12	0.06	0.07	0.03

[a] Data times 10^3 give the number of cells/mm^3.

[b] Underlined values are significantly different from controls ($p < 0.05$).

treatment groups (Table 4). The number of lymphocytes and monocytes appeared low relative to control values for animals exposed to the high concentration for 30 days, although these changes were not statistically significant. After 90 days of exposure, animals from the group exposed to the high concentration had significantly fewer total white blood cells (WBCs) than controls, due primarily to reductions in lymphocytes. In addition, there was a dose-related decrease in the number of monocytes and eosinophils; significant effects were observed for all three treatment groups. The number of neutrophils was unaffected by the exposure. Relative to the 30-day animals, there were increases in the number of leukocytes, lymphocytes, eosinophils, and monocytes in samples from animals in the 90-day exposure group; this difference was unexpected and may have been due to an outbreak of Sendai virus that occurred during the exposure period.

TABLE 5. MEAN SERUM CHEMISTRY PARAMETERS FOR FEMALE RATS AFTER EXPOSURE TO HEAVY DISTILLATE.

TABLE 5. Mean Serum Chemistry Parameters for Female Rats After Exposure to Heavy Distillate

	Protein	Albumin (g/100 ml)	Globulin	Cholesterol	Triglycerides (mg/100 ml)	BUN	SGPT (IU/L)	LDH (IU/L)	Bilirubin (mg/100 ml)
30-DAY EXPOSURE									
Control	5.22	3.74	1.48	71.4	136.7	24.4	38.8	319.8	0.094
Low	5.37	3.97	1.40	79.4	162.8	23.7	45.8	396.2	0.097
Mid	5.34	3.88	1.46	92.4	148.9	22.4	39.4	510.6	0.078
High	5.61	4.10[a]	1.51	116.7	152.4	25.3	41.0	423.6	0.189
90-DAY EXPOSURE									
Control	5.64	3.84	1.80	80.5	114.4	25.1	90.7	788.7	0.067
Low	5.67	3.89	1.78	81.6	126.4	24.7	64.2	743.2	0.075
Mid	5.59	3.91	1.68	99.3	107.1	25.1	55.2	909.3	0.062
High	4.81	3.35	1.46	83.1	146.8	31.9	49.1	325.9	0.041

[a]Underlined values are significantly different from controls ($p < 0.05$).

Blood samples from exposed animals were also evaluated for changes in serum chemistry. Data for female rats are shown in Table 5. After 30 days of treatment, there was an increase in albumin concentration for animals in the high-exposure group. Following 90 days of exposure, total protein concentration from animals in the high-exposure group was significantly lower than controls; reduced concentrations of both albumin and globulin contributed to these lower levels. Similar effects were observed for male rats after 90 days of exposure. Blood urea nitrogen (BUN) concentrations were similar for all groups of animals after 30 days of exposure; however, BUN levels were elevated in the high-dose group following 90 days of treatment. The activity of serum glutamate-pyruvate transaminase (SGPT) was unaffected by 30 days of exposure to HD; however, after 90 days of exposure, the activity of this enzyme decreased in a dose-dependent manner with significant effects observed for all three exposure groups. The activity of lactic dehydrogenase (LDH) was elevated relative to controls for animals in the mid-dose group following 30 days of exposure; this response was not dose-related. After 90 days of exposure, LDH activity decreased dramatically for animals in the high-exposure group. After 30 days of exposure, there were significant increases in the concentration of cholesterol for female rats in the mid- and high-dose groups. Following 90 days of exposure, cholesterol levels for females in the mid-dose group were significantly higher than for controls; however, increases in cholesterol levels were not observed in animals in the high-dose group. Significant increases in cholesterol levels were observed

for males exposed to the high concentration after 30 days of exposure, and in males exposed to the mid concentration after 90 days of treatment. Triglyceride levels were significantly elevated for animals in the high-dose group after 90 days of exposure, but not after 30 days of treatment. Changes in bilirubin concentrations were not observed for animals exposed for 90 days.

DISCUSSION

Exposure to SRC-II HD resulted in a dose-dependent reduction in the growth rate of rats with elimination of growth throughout the exposure period for animals in the high-exposure group. Even though growth was severely retarded for these animals, 90 - 95% of the rats survived 90 days of exposure. In addition to effects on growth and survival, further evidence of toxicity was indicated by changes in tissue weights. Increases in liver weight were apparent after 30 and 90 days of exposure. This change in liver size was observed in the low-dose group, and probably reflects an attempt by the rat to increase excretion rates for HD-derived compounds by increasing their water solubility. The decreases in thymus weight apparent in the high-exposure groups after 30 days of exposure progressed so that significant differences were found for the low-dose group after 90 days of exposure relative to the controls. The biological significance of a decrease in thymus weight is difficult to ascertain (Riley, 1981). Involution of this organ is known to occur in response to stress; however, the thymus is also associated with response of the immune system and these changes may reflect alterations of the defense mechanisms of the animal.

Changes in hematology include effects on both WBC and RBC lines. Decreases in erythrocyte-related measurements were apparent after 30 days of exposure, but were more pronounced following 90 days of exposure. These changes may be due to destruction of circulating RBCs, chronic hemorrhaging, or HD exposure may prevent maturation of RBCs; however, increases in the number of reticulocytes and nucleated RBCs suggest increased erythropoiesis. The results obtained from these data do not allow us to distinguish between these possibilities.

Changes in the leukocyte profile after HD exposure were similar to those observed for animals exposed to benzene (Brief et al., 1980), which is a component of HD. Reports in the literature indicate decreases in total WBC and lymphocyte counts at doses as low as 0.16 mg/L (50 ppm). The benzene concentration in the high-exposure chamber was 0.11 µg/L, which is a factor of 1,450 less than the lowest benzene concentration reported to cause effects on WBCs. These data suggest that the effects observed in this study are not attributable to benzene exposure.

Evaluation of clinical chemistry parameters failed to identify any tissue-specific damage attributable to the exposure; however, this interpretation will require confirmation by histopathologic examination. The pattern of changes observed for the exposed animals is suggestive of a dose-dependent reduction in food intake. This interpretation is supported by reduced concentrations of serum proteins, albumin, globulins, SGPT, and LDH, and increases in the levels of BUN, cholesterol, and triglycerides. Increases in bilirubin levels may be the result of accelerated RBC destruction, although damage to the liver could also account for these changes.

Comparison of the exposure concentrations used in this study with other similar materials provides a better perspective to our data. Heavy distillate, the liquid boiling from 550° - 850°F from the SRC-II process, is composed of 6% aliphatics, 40% polyaromatic hydrocarbons (PAHs), 35% nitrogen-containing PAH, and 14% hydroxylated PAH. Although there are very low concentrations of one- and two-ring PAHs (such as benzene and naphthalenes) in HD, the primary PAHs present are three- to six-fused rings (such as phenanthrene, pyrene, and benzopyrene). Studies on the effects of petroleum hydrocarbons have been conducted by Carpenter et al., (1977) in a series of subchronic inhalation exposure experiments. The materials tested by these workers boiled from 150° - 500°F, and usually the aromatic portion was composed of a high percentage of alkylated benzene compounds. For Carpenter's studies, the aerosol concentrations ranged between 0.1 and 10 mg/L. Significant biological effects attributable to the exposure material were rare and usually not dose related. Of the 16 materials evaluated by these workers, there is a surprising lack of evidence for effects on blood or serum chemistry parameters, even though the exposure concentrations were considerably higher than those used in our study. This lack of correlation of the biological effects for HD and other materials is probably related to the larger molecular weights of the compounds present in HD. Thus, the increased toxicity of HD relative to petroleum materials appears attributable to the larger molecular weights of the compounds present. Alternatively, the concentration of nitrogen- or oxygen-containing PAHs may contribute to the increased toxicity.

The aerosol concentration used for the high-exposure group of this study is large relative to anticipated human exposures; however, certain effects were observed in the low-exposure group and this dose must be considered as an effective dose. For comparison, 0.03 mg/L is six times the threshold limit value (TLV) for exposure to nuisance dust and a factor of 150 times greater than the TLV for coal tar pitch volatiles (American Conference of Governmental Industrial Hygienists, 1971). Even though HD is clearly toxic to the rat when administered by inhalation, conformance to limits similar to those for coal tar pitch volatiles should result in human doses below those which we have found to cause effects in animals.

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PAPER NO. 22

EFFECT OF STODDARD SOLVENT ON KIDNEY FUNCTION AND STRUCTURE OF FISCHER 344 AND SPRAGUE-DAWLEY RATS

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INTRODUCTION

Previous subchronic vapor inhalation studies with a number of light hydrocarbon materials have shown a pattern of kidney damage in male rats (Carpenter et al., 1975a, 1975b, 1975c, 1975d). The kidney damage involved the proximal portion of the tubule. The changes were described as regenerative tubular epithelium and dilated tubules containing proteinaceous material. Following analyses of these studies a number of questions were raised and a program was developed to address these questions. Part of that program consisted of an 8-week inhalation study with Stoddard Solvent. The purposes of the study were to:

1. Determine whether structural changes were accompanied by changes in renal function.
2. Determine whether rats recovered from the kidney damage after a period of non-exposure.
3. Identify the time to onset of the lesion.
4. Identify the characteristics of the lesion at times earlier than previously examined.
5. Identify a strain of rat suitable for chronic testing of light hydrocarbons.

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The major emphasis of this study was on the clinical assessment of renal function and its comparison to structural damage. Analyses were chosen for their simplicity and non-invasiveness (Berndt, 1981; Sharratt and Frozer, 1963; Diezi and Biollaz, 1979; Balazs et al., 1963; Kluwe, 1981a). Because of the design, the same tests could also be applied as routine screening tests for humans. Individually, these tests are not the most sensitive. However, when conducted properly and analyzed together, they produce very useful information. To further enhance the usefulness of these methods, the analyses were quantitative measurements and not the routine qualitative (dip-stick) measurement (Kluwe, W.M., 1981b).

METHODS

Male and female Sprague-Dawley and Fischer 344 rats were divided into two groups of 50/sex/strain/exposure level. The two groups were exposed to Stoddard Solvent vapor at approximate concentrations of either 4.58 mg/L (800 ppm) or 0.57 mg/L (100 ppm) for 6 hours per day, 5 days per week, for 8 weeks. An additional group of rats, identical to the treatment groups, was exposed to chamber air only and used as controls. Following exposure days 2, 5, 20, 40, and a period of 4 weeks without exposure (recovery), 10 rats/sex/strain/group predesignated were sacrificed immediately following blood collection and an overnight urine collection. At sacrifice, specific organs were weighed and preserved for histopathology.

An additional group of rats, 10/sex/strain/exposure level, and controls, were exposed throughout the 8 weeks and maintained through the 4-week recovery period. These rats were used for urine concentration tests following 1, 4, and 8 weeks of exposure and following a 4-week observation period. No other clinical or pathological analyses were made in this segment of the study.

RESULTS

A complete analysis of both the kidney function and kidney pathology data revealed that the most significant and meaningful effects in relation to light hydrocarbon nephrotoxicity were noted in the male Fischer 344 rat. This group showed a pattern of change in kidney function that correlated well with structural damage. The male Sprague-Dawley rat developed a pattern similar to the Fischer 344 male, but the response was not as well defined. A few statistically significant changes were observed in indices of kidney function in the female Fischer 344 and Sprague-Dawley rats. However, these changes were considered to have little if any significance with regard to nephrotoxicity.

In the male Fischer 344 rat urine, volume was significantly increased after 8 weeks in the 4.58 mg/L group, while urine concentration (osmolality) was significantly decreased (Table 1). Male Fischer 344 (F344) rats exposed for 4 to 8 weeks to both 0.57 and 4.58 mg/L of Stoddard Solvent, from the urine concentration test,had significantly lower osmolalities than controls (Figure 1).

TABLE 1. URINE VOLUME AND OSMOLALITY FROM MALE FISCHER 344 RATS EXPOSED TO STODDARD SOLVENT VAPOR

	Volume (ml/16 hr)			
	1 Week	4 Weeks	8 Weeks	Recovery
CONTROL	6.2 ± 0.6	11.4 ± 1.5	9.4 ± 1.0	6.6 ± 0.9
0.57 mg/L	6.5 ± 1.2	13.3 ± 1.8	11.6 ± 1.5	9.7 ± 1.5
4.58 mg/L	7.0 ± 1.4	15.5 ± 1.8	19.8 ± 1.5*	8.6 ± 1.3
	Osmolality (mOsm/kg)			
CONTROL	861 ± 80	639 ± 95	685 ± 78	1486 ± 192
0.57 mg/L	864 ± 114	579 ± 94	682 ± 121	1001 ± 135
4.58 mg/L	996 ± 115	541 ± 54	445 ± 22*	1172 ± 180

*$p < 0.05$

Urine glucose and protein were increased about 4 and 8 weeks in both the low and high exposed F344 male rats (Figures 2 and 3). Renal epithelial cell exfoliation was increased following 8 weeks in both exposure groups of male F344 rats (Table 2). Although creatinine clearance was not affected (Table 3), serum creatinine levels of male F344 rats exposed to 4.58 mg/L of Stoddard Solvent were slightly increased following 4 and 8 weeks of exposure (Table 4). Following 4 weeks of recovery, there were no differences between exposed and control male F344 rats for any of the clinical measurements.

Structurally, the kidneys of the male F344 rats exposed to Stoddard Solvent exhibited regenerative tubular epithelium and tubules dilated with granular material at the cortico-medullary junction. The structural lesions were observed following 4 and 8 weeks of exposure and increased in severity and incidence with dose and time of exposure. Structural recovery following the 4-week recovery period was not complete, although indicated. Other statistically significant effects on the kidney of male Fischer 344 rats included an increase in kidney weight in the high exposure group following exposure weeks 4 and 8, and an increase in kidney weight to body weight ratio in the high exposure group following exposure weeks 1, 4, and 8 (Table 5).

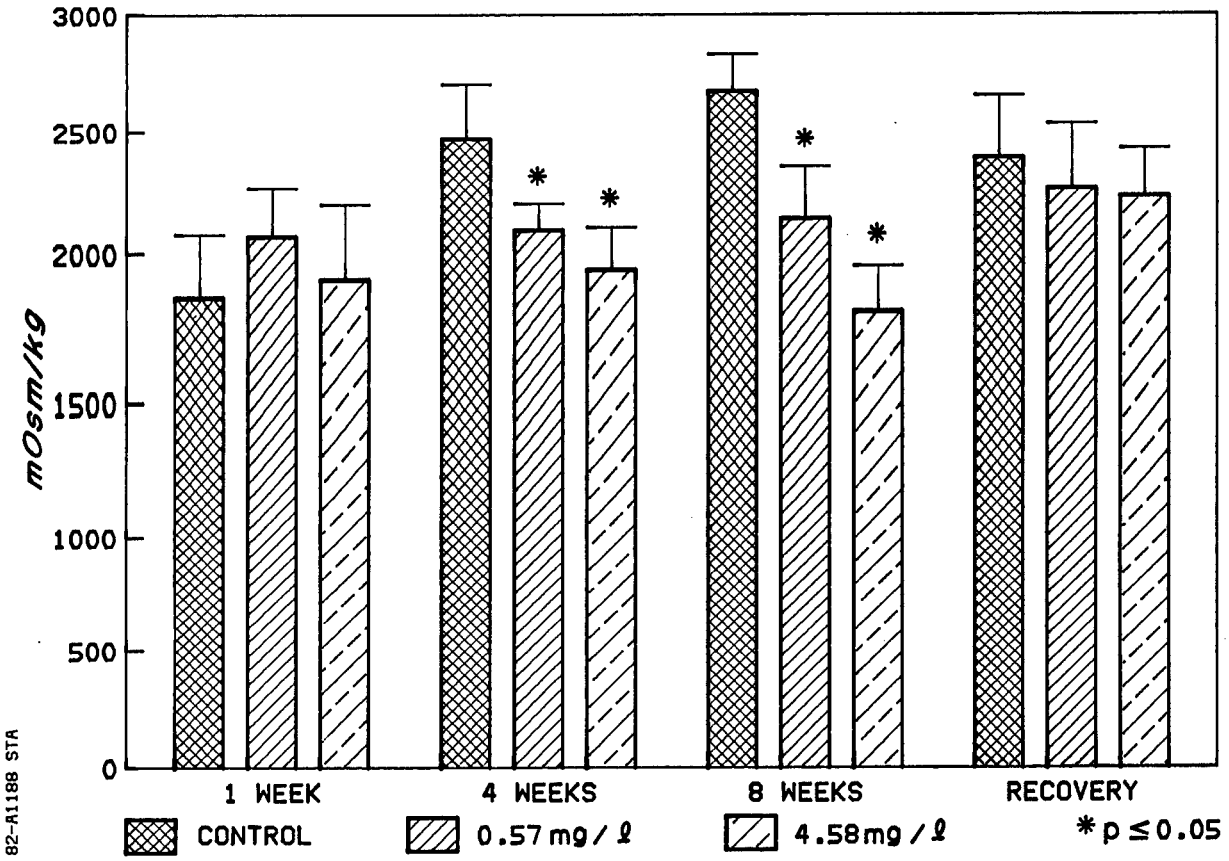

Figure 1. Urine concentration test from male Fischer 344 rats exposed to Stoddard Solvent vapor.

FIGURE 2

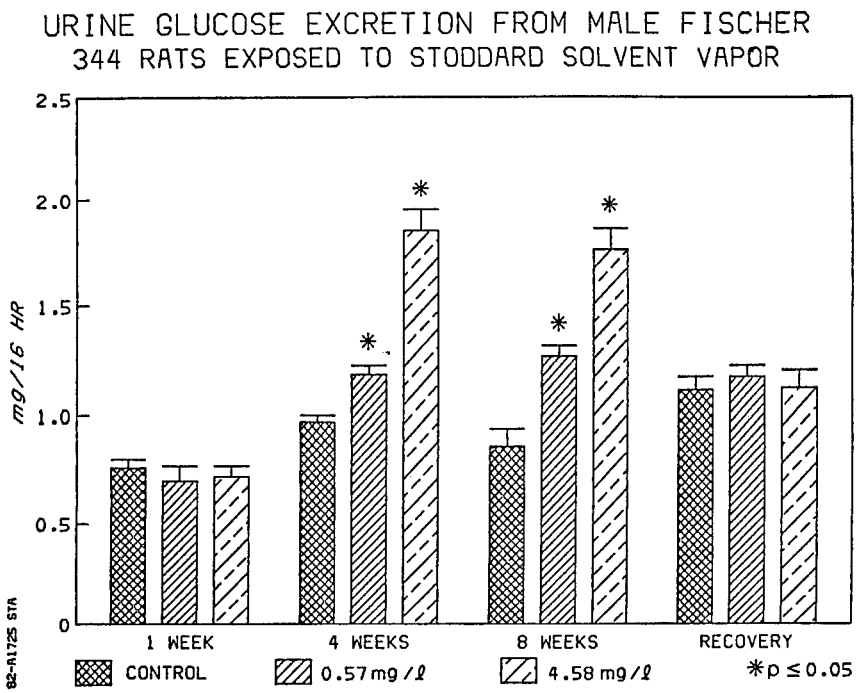

Figure 2. Urine glucose excretion from male Fischer 344 rats exposed to Stoddard Solvent vapor.

TABLE 2. RENAL EPITHELIAL CELL EXFOLIATION FROM MALE FISCHER 344 RATS EXPOSED TO STODDARD SOLVENT VAPOR

	8 Weeks	Recovery
CONTROL	1,314 ± 410	2,490 ± 359
0.57 mg/L	12,752 ± 2,911	2,257 ± 424
4.58 mg/L	65,231 ± 14,145	2,291 ± 424

TABLE 3. CREATININE CLEARANCE FROM FISCHER 344 RATS EXPOSED TO STODDARD SOLVENT VAPOR

	1 Week	4 Weeks	8 Weeks	Recovery
CONTROL	0.61 ± 0.12	1.04 ± 0.12	0.98 ± 0.22	1.19 ± 0.23
0.57 mg/L	0.57 ± 0.16	1.01 ± 0.07	1.04 ± 0.12	1.30 ± 0.20
4.58 mg/L	0.63 ± 0.15	0.95 ± 0.11	1.02 ± 0.21	1.23 ± 0.23

TABLE 4. BUN AND SERUM CREATININE FROM MALE FISCHER 344 RATS EXPOSED TO STODDARD SOLVENT VAPOR

BUN (mg/dl)

	4 Weeks	8 Weeks	Recovery
CONTROL	16.0 ± 0.5	15.6 ± 0.4	17.7 ± 0.5
0.57 mg/L	16.4 ± 0.4	16.5 ± 0.4	16.7 ± 0.5
4.58 mg/L	17.2 ± 0.6	18.3 ± 0.6	17.8 ± 0.6

Creatinine (mg/dl)

CONTROL	0.58 ± 0.02	0.63 ± 0.02	0.63 ± 0.02
0.57 mg/L	0.59 ± 0.02	0.65 ± 0.02	0.62 ± 0.01
4.58 mg/L	0.68 ± 0.02	0.77 ± 0.02*	0.64 ± 0.01

*$p<0.05$

With regard to the male Sprague-Dawley rat, a number of statistically significant differences were observed in various measurements in the urine analysis. The most significant change was probably the decrease in urine osmolality of the high-dose rats at 8 weeks from the urine concentration test (Figure 4.).

FIGURE 3

URINE PROTEIN EXCRETION FROM MALE FISCHER 344 RATS
EXPOSED TO STODDARD SOLVENT VAPOR

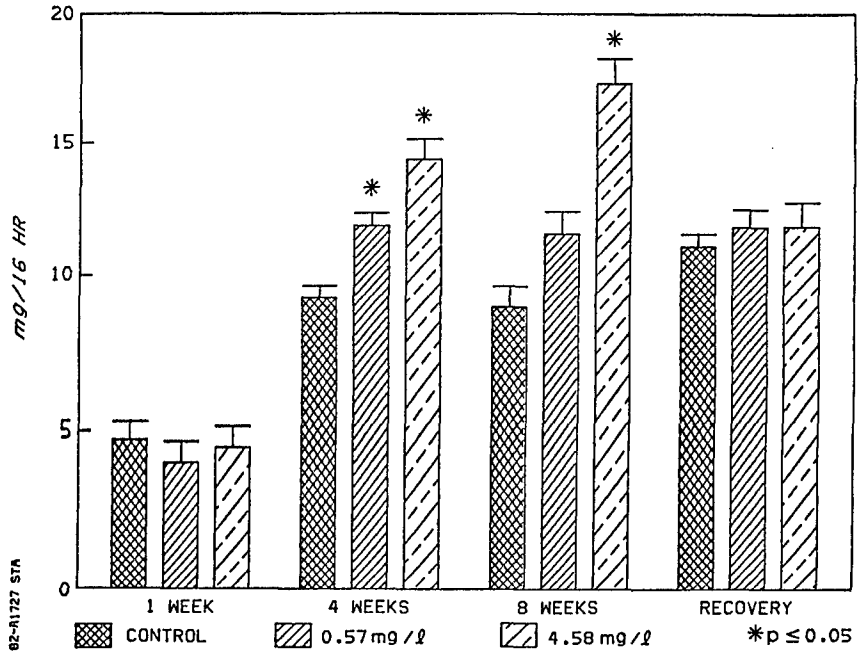

Figure 3. Urine protein excretion from male Fischer 344 rats exposed to Stoddard Solvent vapor.

FIGURE 4

URINE CONCENTRATION TEST FROM MALE SPRAGUE-DAWLEY
RATS EXPOSED TO STODDARD SOLVENT VAPOR

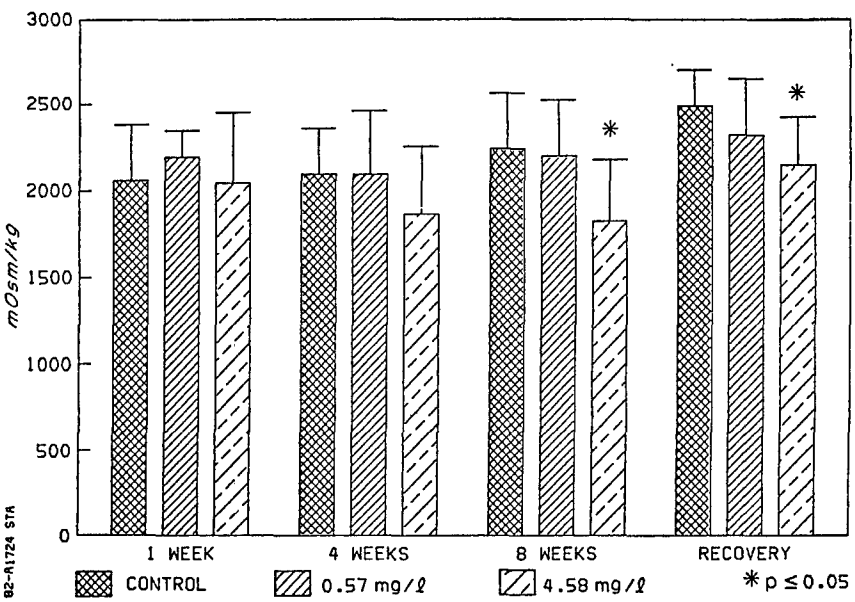

Figure 4. Urine concentration test from male Sprague-Dawley rats exposed to Stoddard Solvent vapor.

TABLE 5. TOTAL KIDNEY WEIGHT AND KIDNEY WEIGHT/BODY WEIGHT RATIO FROM MALE FISCHER 344 RATS EXPOSED TO STODDARD SOLVENT VAPOR

	1 Week	4 Weeks	8 Weeks	Recovery
Kidney Weight				
CONTROL	1.23 ± 0.11	1.62 ± 0.11	1.80 ± 0.10	2.03 ± 0.11
0.57 mg/L	1.30 ± 0.13	1.59 ± 0.09	1.79 ± 0.06	2.04 ± 0.10
4.58 mg/L	1.34 ± 0.12	1.75 ± 0.14*	2.03 ± 0.18*	2.03 ± 0.18
Kidney Weight/Body Weight				
CONTROL	0.84 ± 0.03	0.74 ± 0.02	0.70 ± 0.02	0.69 ± 0.04
0.57 mg/L	0.88 ± 0.05	0.75 ± 0.02	0.67 ± 0.02	0.67 ± 0.02
4.58 mg/L	0.93 ± 0.03*	0.88 ± 0.04*	0.80 ± 0.03	0.71 ± 0.04

*$p<0.05$

Most of the other changes were minimal and not always complementary of each other, as observed with the Fischer 344 male rat. Histopathology of the kidney from the male Sprague-Dawley rats showed the typical changes of increased tubular regenerative epithelia and tubular dilation with intratubular protein. The incidence of these changes in the Sprague-Dawley rats was much less than that observed in the Fischer 344s. This pattern of structural changes agrees with the differences observed in the urine analysis changes between the two rat strains.

Female F344 and female Sprague-Dawley rats exposed to Stoddard Solvent vapor for 8 weeks did not produce a complement of changes typical of nephrotoxicity.

DISCUSSION

Eight weeks of exposure to Stoddard Solvent vapor produced a pattern of changes in the male Fischer 344 rat typical of nephrotoxicity. The changes indicated structural damage to the tubular portion of the nephron, accompanied with a minimal functional decrement. The effect appeared following 4 weeks of exposure, and continued through 8 weeks. The structural damage showed a dose-response relationship. Following a 4-week period of recovery, the functional decrement observed following 8 weeks of exposure was not evident, and the structural changes were much less.

With the exception of the renal cell exfoliation, the magnitude of these changes was not great. For example, significant changes in the 0.57 mg/L group after 4 and 8 weeks of exposure

for glucose, protein, and urine concentration were not quantitatively different from control values observed in the recovery period. The change observed in the control value is probably related to the increasing age of the male rats. Also, the absence of an effect on creatinine clearance would tend to minimize the clinical significance of these changes up to this point (8 weeks) in the exposure history of the animals.

Previously, this kidney damage had been compared to a disease that occurs naturally in aging rats known as Chronic Progressive Nephropathy (CPN) (Barthold, 1979). One possible explanation for the kidney damage was that exposure to light hydrocarbons could hasten the onset of this aging phenomenon. However, two characteristics of the light hydrocarbon-induced change were different from CPN. First, in CPN there is early evidence of glomerular damage (Barthold, 1979). In young rats, this is evidenced by thickening of the glomerular basement membrane. As the disease progresses, glomeruli enlarge due to capillary dilation, resulting in occlusion of Bowman's space. Glomerular changes have not been observed in the kidneys of rats exposed to light hydrocarbon vapors. Secondly, protein accumulates in the tubules of rats with CPN. This protein stains amorphous with H & E, and is probably protein from glomerular filtrate. The protein in tubules of male rats exposed to light hydrocarbons is granular in character, and is probably cellular debris. It would appear then that though the rat is prone to an aging phenomenon in the kidney, this phenomenon is different from that observed in male rats exposed to light hydrocarbons.

In conclusion, this study indicates that at exposure levels as low as 0.57 mg/L, repeated exposure is needed to elicit the nephrotoxic response in male rats. An acute nephrotoxic response following 2 and 5 exposures at levels up to 4.58 mg/L was not observed in this study. Since this study was only 8 weeks in duration, it may not adequately characterize the eventual effect resulting from longer-term exposure to rats.

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until 1977. That was when President Carter cancelled the B-1 bomber in favor of developing the long-range, strategic air-launched cruise missile (ALCM). Other cruise missiles of interest are the HARPOON, an anti-ship missile, and the Tomahawk, a ground-to-ground cruise missile. The Navy has a sea-launched version of the Tomahawk, to be used as an anti-ship missile. These new-generation cruise missiles all use special high-density fuels, mostly JP-10, to increase their range. JP-10 by itself allows a 20% increase in range over conventional petroleum-derived fuels. As with more conventional fuels, JP-10 can be expected to occasionally produce environmental contamination from accidents of low-level occupational exposure to workers during handling and testing of the missiles.

JP-10 is the exo isomer of tetrahydrodicyclopentadiene. Chemical Abstracts lists the molecule as a methanoindene[1]. The molecule has also been described as a tricyclodecane (tricyclo-[5.2.1.$0^{2,6}$]decane) and as a trimethylenenorbornane. Several of the high-density fuels have molecular ring structures similar to the cyclodiene chlorinated insecticides. JP-10 has the same carbon skeleton as chlordane and heptachlor. However, the fuels are pure hydrocarbons and contain no chlorine atoms. JP-10 is formed from dicyclopentadiene (DCPD), a by-product of naphtha cracking in the manufacture of ethylene. Hydrogenation of the DCPD produces the solid material endo-tetrahydrodicyclopentadiene (referred to hereafter as the endo isomer of JP-10). The endo isomer is the major impurity in JP-10 (about 2%). The endo-structure is catalytically isomerized to exo-tetrahydrodicyclopentadiene, or JP-10 (Figure 1). Further rearrangement of the molecule can be accomplished catalytically to form adamantane, a common component of perfume (Luke, 1971).

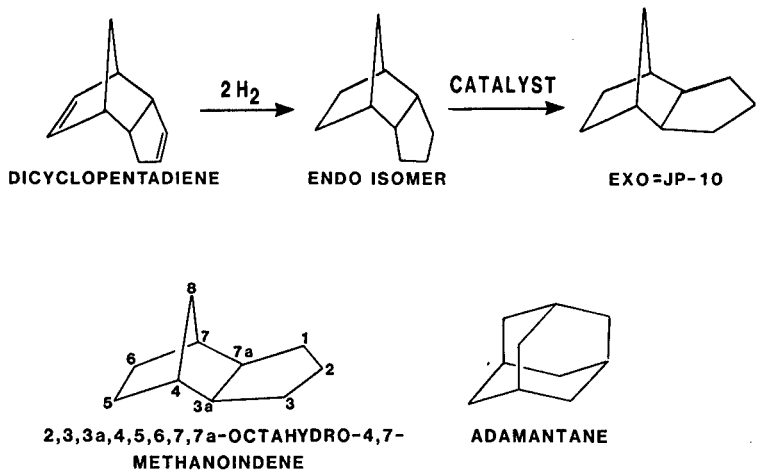


Figure 1. STRUCTURE OF JP-10 AND RELATED COMPOUNDS

Figure 1. Structure of JP-10 and related compounds.

[1]2,3,3a,4,5,6,7,7a-octahydro-4,7-methanoindene, Registry No. 2825-83-4.

In 1973, the Air Force Aero Propulsion Laboratory at Wright-Patterson AFB, OH, contracted with the Sun Oil Company to prepare at least five candidate hydrocarbon fluids having net volumetric heats of combustion and low temperature viscosities superior to those of Shelldyne-H, the current leading high-density missile fuel for ramjet and/or turbine applications. This contract resulted in a three-volume publication (Schneider, 1974). JP-10 was one of the candidate high-density fuels listed in the report. As performance testing at the fuels laboratory was completed, several of the more promising candidate fuels were subjected to acute toxicity testing. These included RJ-5 (Shell-dyne-H, a norbornane dimer), RJ-4 (a dimethyl JP-10), JP-10, and others (Burdette, 1978). Table 1 compares some of the physical properties of these high-density fuels with JP-4, a petroleum distillate fuel.

TABLE 1. TYPICAL PROPERTIES OF MISSILE AND AIRCRAFT JET FUELS

Comparison of a Petroleum Distillate Fuel (JP-4) with 3 High-Density Fuels

	JP-4	RJ-4	RJ-5	JP-10
Heating Value, BTU/gal	118,000	140,000	161,000	142,000
Viscosity, cSt @ 40°F	4.5	60	2,000	19
Freezing Point, °F	-72	-40	0	-110
Flash Point, °F	-20	150	1.08	135
Specific Gravity @ 60°F	0.77	0.94	1.08	0.94

Physical/Chemical Characteristics of JP-10

Vapor Pressure at 74°F = 1.6 Torr -----→ 2130 ppm at Saturation
Molecular Weight = 136
Specific Gravity = 0.94 at 60°F
Boiling Point = 360°F
Freezing Point = -110°F

Acute toxicity studies with JP-10 were performed at the Toxic Hazards Research Unit in 1978 after the fuel had been identified as a likely candidate for use in the ALCM. Once selection of JP-10 was a certainty, a one-year intermittent inhalation exposure was initiated to test for potential oncogenic effects. The pathology results from this study are only now becoming available. The acute toxicity studies (Kinkead, 1979) demonstrated that JP-10 is only slightly toxic to rats, mice, and hamsters. The acute oral

LD_{50}'s in rats (male and female Fischer 344) and hamsters were unattainable since the maximum volume dose (20 ml/kg) produced no deaths in either species. Mice (female C57B1/6) were more sensitive, and an acute oral LD_{50} was established at 3.9 ml/kg. Deaths, with convulsions immediately preceding death, occurred within 48 hours of dosing. The IP LD_{50} (ml/kg) was 1.2 in male rats, 1.6 in female rats, 1.4 in male hamsters, and 1.1 in female mice.

The 4-hour inhalation LC_{50} in male and female rats was 1200 ppm, and approximately 925 ppm in female mice. There was essentially no sex-related difference in the acute inhalation toxicity in rats. There were no deaths in hamsters at 1500 ppm. Male Sprague-Dawley rats exposed by inhalation to a maximum obtainable JP-10 concentration (approximately 1500 ppm) convulsed in about an hour. Several of those that did not die in convulsions were left with posterior paralysis at the end of the two-hour exposure period. Gross pathology findings in the animals that died were limited to multifocal congestion and areas of emphysema scattered throughout the lungs. The livers were occasionally swollen with a reticulated appearance.

In inhalation studies with dogs, four animals were exposed to 718 ppm JP-10 for 30 minutes. This concentration produced slight lacrimation in the dogs. One of the dogs had a violent coughing spell after 25 minutes of exposure but this subsided and the dog appeared normal. These animals performed previously learned tasks satisfactorily after exposure. Neurological examinations were normal. Hematology values at 14 and 28 days postexposure were normal. Exposure of dogs to 1000 ppm for 10 minutes produced similar results except that mild eye irritation appeared sooner (2 minutes), and two of the dogs displayed fine tremors at the end of the exposure period.

Results of irritation tests in rabbits were negative. JP-10 (no dilution) was not irritating to the eyes or to either abraded or intact skin of rabbits after 24, 48, or 72 hours. Eight of 20 guinea pigs showed a mild sensitization response to JP-10.

Lyng (1981) found no evidence of embryotoxicity when pregnant ICR mice were treated orally with 0.2, 0.4, 0.6, and 0.8 mg/kg JP-10 during the period of organogenesis. Keller (1981) orally dosed female rats with 250, 500, or 1000 mg JP-10/kg/day on gestation days 6 through 15. Inhalation exposures for this study were at 600 ppm, 6 hours/day for 10 days. Approximately one-half of each litter was examined for soft tissue malformations and the remaining fetuses were examined for skeletal malformations. The 1000 mg/kg oral dose group and the inhalation exposure group had significantly reduced maternal weight gains. Convulsions were seen in the inhalation group, and tremors occurred in the high oral dose group. However, fetal weights and incidence of malformations and resorption were not significantly different from the controls.

A battery of 5 in-vitro, short-term assays was performed on JP-10 to assess its biological activity (Little, 1982). The assays conducted included the Ames Salmonella/mammalian microsomal mutagenicity assay, the CHO/HGPRT[2] gene mutation assay, the CHO/sister chromatid exchange assay, the CHO/chromosome aberration assay, and the BALB/c-3T3 neoplastic transformation assay. The JP-10 had a marginal clastogenic effect in the CHO/chromosome aberration assay. In the other assays, there were negative or inclusive results because of solubility problems or cytotoxicity of the fuel; for example, in the Ames test, even the lowest dose tested (1 µl/plate) produced sparse background growth when added directly to the bacteria and S-9 or saline mixes for pre-incubation. The pre-incubation technique was used because the JP-10 was immiscible with the agar medium.

METHODS

GAS LIQUID CHROMATOGRAPHY

In our studies, gas-liquid chromatography was used to quantitate the JP-10 synthesized potential metabolites and extracted components from biological samples. N-Hexane was the solvent of choice. Methyl silicone capillary columns and SE-30 packed columns provided good separations. For the capillary chromatogram of JP-10 shown in Figure 2, a split injection was used. Temperature was programmed from 100° to 200°C. The first large peak is the hexane solvent and the next is JP-10. The endo isomer closely follows the JP-10 peak, and then 2 minor impurities follow.

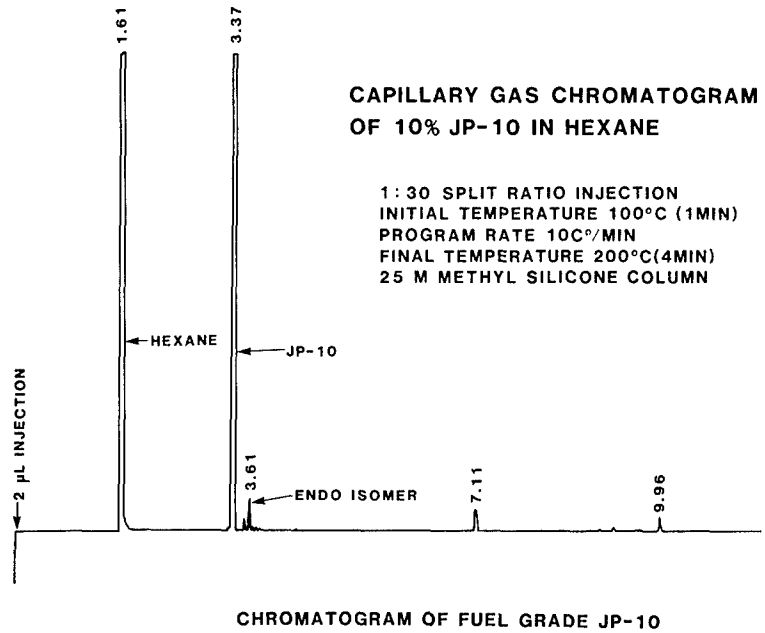


Figure 2. Chromatogram of fuel grade JP-10.

[2]CHO/HGPRT = Chinese hamster ovary/hypoxanthine-guanine phosphoribosyltransferase.

EXTRACTION OF METABOLITES FROM URINE

In our attempt to isolate and identify JP-10 and its metabolites in the urine, we tried many different extraction techniques. The method shown in Figure 3 using a pH of 6.8 was found to be optimum for this technique. Urine samples were treated with beta-glucuronidase and sulfatase and placed onto a silica column (Clin Elut). Metabolites were eluted from the column with hexane. A concentration step then readied the sample for gas chromatography and mass spectrometry.

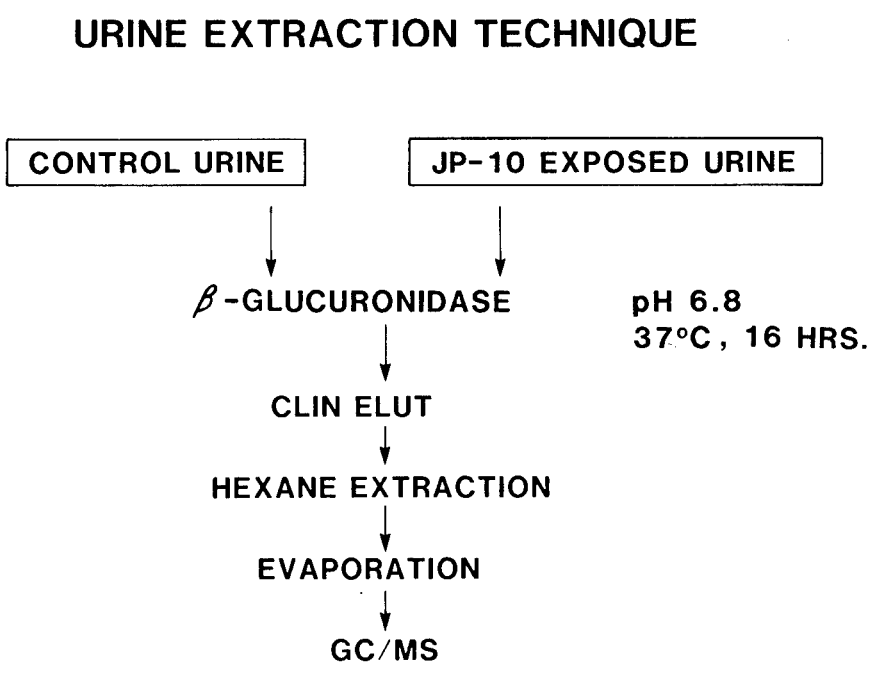


Figure 3. Urine extraction technique.

GAS CHROMATOGRAPHY/MASS SPECTROMETRY (GC/MS)

Mass spectral analysis of JP-10 and its metabolites was performed using a Hewlett-Packard 5985 GC/MS. Most studies employed electron impact ionization but in some special cases chemical ionization was used. The mass spectrum at the top of Figure 4 is that of JP-10. Notice the prominent molecular ion peak at M/Z 136. M/Z 95 (M-41) is the base peak and probably represents a loss of C_3H_5. The fragmentation behavior of JP-10 and its possible metabolites had not been described previously. Therefore, the authors performed extensive studies to elucidate the fragmentation patterns in order to fully utilize the full power of mass spectral analysis. Mass labelling was used to study the fragmentation of JP-10 under electron impact. Starting with dicyclopentadiene, JP-10 was synthesized with deuterium in the X positions, then on the opposite side of the molecule in the Y positions and, finally, in all four positions (Figure 5). By comparing the abundance of the resulting fragmentations, we were able to describe two generic fragmentation patterns. One involved the bisection of the molecule to produce M/Z 68 ions; the other showed the loss of various alkyl fragments (Inman, 1982).

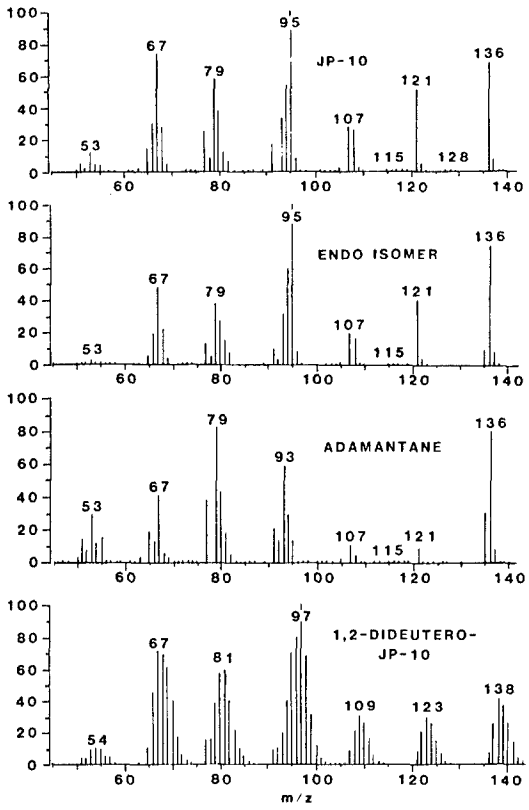
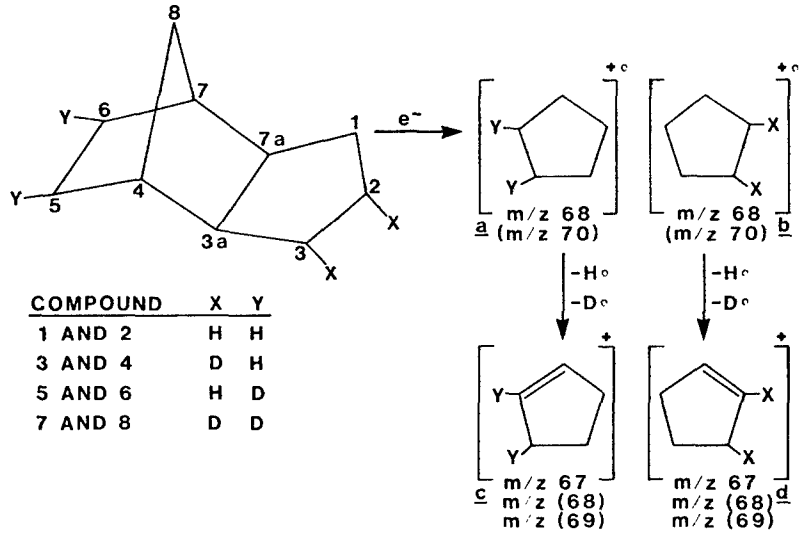


Figure 4. MASS SPECTRA OF JP-10 AND RELATED COMPOUNDS

Figure 4. Mass spectra of JP-10 and related compounds.



COMPOUND	X	Y
1 AND 2	H	H
3 AND 4	D	H
5 AND 6	H	D
7 AND 8	D	D

Figure 5. FRAGMENTATION OF 1-8 LEADING TO BISECTING OF THE MOLECULE. MASS NUMBERS IN PARENTHESES ARE FOR X = Y = D

Figure 5. Fragmentation of 1-8 leading to bi-secting of the molecule. Mass numbers in parentheses are for X=Y=D.

RADIOLABELLED JP-10

Kinetic studies were performed using carbon-14 labelled JP-10. The radiolabelled fuel was synthesized by Amersham. The C-14 carbon is in the No. 2 position on the molecule. Specific activity was 206 μCi/mmol. Tritium labelling was unsuitable for metabolism studies once we discovered that oxidation occurred at the sites of tritium attachment and therefore would result in the loss of the label. In the kinetic studies, male Fischer rats were injected intraperitoneally with 0.3 ml/kg of the radiolabelled JP-10. The rats were then placed in Roth metabolism chambers to measure excretion of the label in expired air, urine, and feces. Samples were taken at intervals over a 50-hour period. JP-10 was trapped from expired air using the Carbosorb, a liquid/amine chemical produced by Packard Instruments. At the end of the 50-hour sampling period, the animals were sacrificed using an overdose of Halothane. Twenty tissues were collected and weighed to determine radioactivity per gram of tissue. Tissue samples were prepared for counting by incineration in a Packard 306 Sample Oxidizer. This instrument oxidizes the sample in an oxygen atmosphere so that the carbon-14 can be trapped as radiolabelled CO_2. Beta emissions from all samples were measured with a Packard Tri-Carb 2660 liquid scintillation system.

RESULTS

ANALYSIS OF URINE

A major metabolite, 5-hydroxy JP-10, was found in the urine of male rats exposed to JP-10 when the urine was incubated with beta-glucuronidase and extracted with hexane. Figure 6 is the mass spectrum of this metabolite. No unmetabolized JP-10 was found in the urine. Figure 7 demonstrates that the metabolite is excreted in the urine mainly as a glucuronide. In this study, four male rats were exposed to JP-10 by inhalation. Their urine was

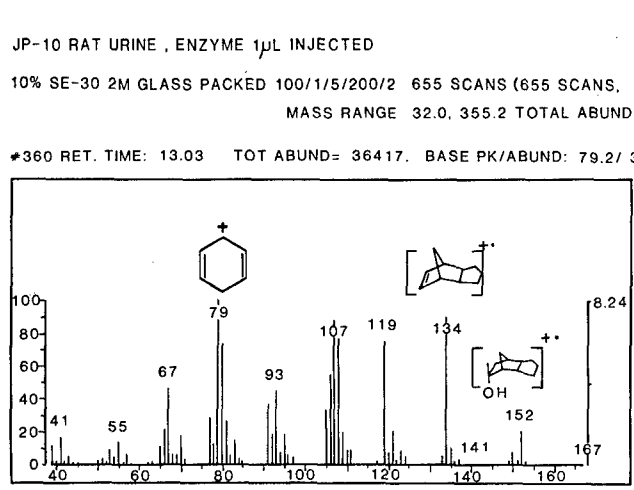


Figure 6. 5-Hydroxy-exo-JP-10.

collected and pooled from metabolism cages in which ice was packed around the urine collection flask to retard bacterial action in the urine. Fifteen ml of this urine was incubated with beta-glucuronidase for 18 hours at 37°C. Extraction was performed on the enzyme-treated urine and on an equal amount of the same urine pool that had been kept refrigerated. Figure 7 illustrates the

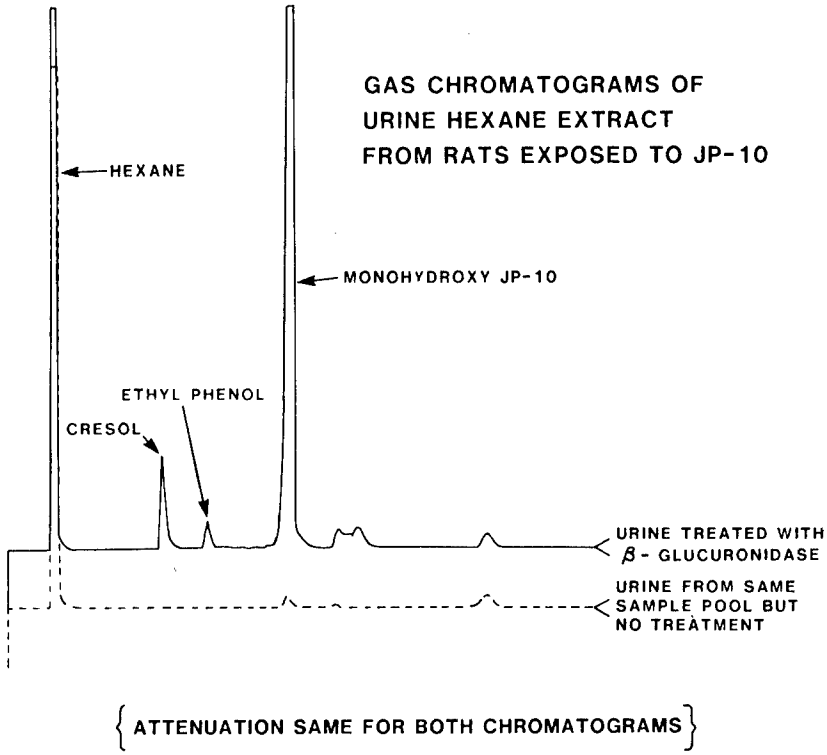


Figure 7. Gas chromatograms of urine hexane extract from rats exposed to JP-10.

results of enzyme treatment by superimposing the chromatogram of the treated urine upon that of the untreated urine. Peak identification was by mass spectrometry. Three major peaks appeared in the enzyme-treated urine that were very small or non-existent in the untreated urine. They were cresol, ethyl phenol, and monohydroxy JP-10. The area under the monohydroxy JP-10 peak is more than 100 times larger than the corresponding untreated urine peak. The hydroxyl group is on the No. 5 carbon and the peak is a mixture of endo- and exo-hydroxy JP-10. The phenolic compounds appear normally in urine of control rats if their urine is treated with glucuronidase. No JP-10 or keto JP-10 was ever found in the urine in any of the methods development studies for this research.

IDENTIFICATION OF MAJOR METABOLITES

To study the metabolism of JP-10, we had to synthesize potential metabolites (alcohols and ketones) since none of these chemicals had been reported in the literature. We have published the details of the synthesis in the Journal of Organic Chemistry, 47:4348 (1982). Nineteen different possible metabolites were synthesized. The mass spectrum of the most abundant metabolite we have found in urine is shown in Figure 6. This metabolite has a single hydroxyl group on the No. 5 showing up well on the mass spectrum.

TISSUE DISTRIBUTION OF JP-10

To study the tissue distribution of JP-10, the C-14 radiolabelled fuel was administered by intraperitoneal injection. Four male rats were exposed to radiolabelled JP-10, and the tissues sampled 50 hours later. The radioactivity was expressed as μmol of JP-10 per gram of tissue in order to normalize the data. Since all of the rats could not be administered JP-10 with the same specific activity, the normalization procedure was accomplished so that comparisons could be made. However, the units of μmol JP-10/g of tissue do not imply that the radioactivity represents JP-10. Certainly, at 50 hours after exposure the radioactivity represents mostly metabolites rather than JP-10.

The relative distribution of radioactivity in the four male rats given radiolabelled JP-10 intraperitoneally is shown in Table 2. Kidney and retroperitoneal fat from near the kidney had higher concentrations of radioactivity than any other tissue not associated with the path of bile through the gut. High concentrations in the kidney and fat near the kidney are similar to the findings of Withey (1979) for styrene monomer. The Mann-Withey test was used to compare kidney and liver concentrations. These data show the kidney to have significantly higher concentrations of radioactivity than the liver.

DISCUSSION

This research was initiated for the purpose of obtaining toxicity information that would be beneficial in evaluating the occupational and environmental hazards of JP-10 beyond the data provided by routine toxicity tests. Acute exposure situations in which humans are exposed to very high concentrations of JP-10 vapors are extremely unlikely to occur in the workplace. A more likely exposure situation would involve low-level exposures of less than 100 ppm for long periods of time, and/or skin contamination. JP-10 has a very pungent odor that would not be voluntarily tolerated in high concentrations. Because of its low vapor pressure (1.6 Torr), JP-10 would not be expected to produce high

TABLE 2. TISSUE CONCENTRATIONS OF RADIOACTIVITY AS *JP-10 (μmol/gm) 50 HOURS AFTER I.P. INJECTION[a]

Tissue[b] μmol/gm	*JP-10[c]
Cecal Contents	8.05
Large Intestine Contents	7.77
Small Intestines	1.50
Feces	1.45
Retroperitoneal Fat	1.39
Large Intestine	1.32
Kidney	0.86
Epididymal Fat Pad	0.67
Omentum	0.65
Stomach Contents	0.33
Liver	0.30
Lung	0.07
Testis	0.06
Quadriceps Muscle	0.06
Spleen	0.06
Heart	0.06
Whole Eye	0.05
Cerebrum	0.04
Cerebellum	0.03

[a]Dose = 0.3 ml/kg

[b]Listed in order of decreasing concentration

[c]Mean values, N = 4

room air concentrations. During initial studies, the actual highest concentration measured in a sealed flask at equilibrium (room temperature) was approximately 600 ppm. This was the basis for selecting 600 ppm as representative of a practical worst-case human exposure situation. A concentration of 1500 ppm was the highest concentration that could be generated in the exposure chambers used for this research.

In initial studies with rats, we found that intravenous injection of JP-10 produced rapid death in the animals,. an effect probably related to an embolus effect due to the fuel's low solubility in aqueous solutions. Thus, traditional half-life and volume-of-distribution studies were impractical.

The behavior of JP-10 in the tissues of the rat is what is expected of a highly lipid, soluble chemical. Partition coefficients were measured using a vial equilibration method[3]. The olive oil - air partition coefficient was 12970, the blood:air value was 62, and the water:air value was 0.49.

During methods development for extracting JP-10 metabolites from urine, the highest efficiency found in extracting the monohydroxy JP-10 was by using a neutral pH extraction with N-hexane, followed by clean-up with a disposable silica column. However, this method produces only a 47% efficiency with the monohydroxy JP-10 and 56% efficiency for the keto JP-10. This and other extraction efficiency problems could indicate the existence of a more polar metabolite, as yet undetected, and/or binding problems. Further studies are indicated and will be initiated using high-performance liquid chromatography coupled with a flow detector for radioisotopes.

The acute toxicity of JP-10 is low. However, recent findings of tumors in male rats during chronic studies are sure to generate concern. Renal toxicity and oncogenicity seem to result from the effects of JP-10 and many other volatile agents on male rat kidneys. This is certainly a topic of major concern for the Air Force and any other organization where humans are occupationally exposed to fuel vapors. Several additional studies are planned or are underway to evaluate the importance of sex-related renal toxicity in rats.

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PAPER NO. 26

EFFECT OF 2,5-HEXANEDIONE ON TESTICULAR MORPHOLOGY AND BIOCHEMISTRY

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INTRODUCTION

n-Hexane is one of a family of hexacarbons which are neurotoxicants in both humans (Yamamura, 1969) and experimental animals (Shaumburg & Spencer, 1976). The severity of the neurotoxicity has been found to be proportional to the amount of a metabolite of hexane, 2,5-hexanedione (2,5-HD), produced in vivo (Krasavage et al., 1980). Oral administration of 2,5-HD will produce peripheral neuropathy (Couri & Nachtman, 1979) and testicular degeneration (Krasavage et al., 1980) similar to that seen after n-hexane treatment of laboratory animals. We used a previously well-characterized drinking water model of 2,5-HD administration (Gillies et al., 1980) to examine the testicular lesion seen in rats after 2,5-HD consumption. This work has been described previously (Chapin et al., 1982; Chapin et al., in press).

METHODS

Adult male Fischer-344 rats (CDF/CrlBR®, 160-180 gr., Charles River, Kingston, NY) consumed a 1% v/v solution of redistilled 2,5-HD in tap water as their sole fluid source (Gillies et al., 1980). Because animals consumed less food during treatment, a pair-fed animal was run with each treated rat to act as a diet-restricted control (Chapin et al., 1982). Animals with ad lib access to feed and water were used as an untreated control group. Plasma levels of testosterone, luteinizing hormone (LH), and follicle stimulating hormone (FSH) were measured by radioimmunoassay using a commercial preparation (testosterone) or kits generously provided by the N.I.A.M.D.D. and Dr. A. F. Parlow (LH and FSH). For histologic evaluation, animals were anesthetized with Metofane® (Pitman-Moore), killed by exsanguination and tissues were fixed in situ by systemic perfusion with Karnovsky's fixative (Karnovsky, 1965). For light microscopy, tissues were embedded in glycol methacrylate, cut at 3 μm, and stained with PAS and hematoxylin. For electron microscopy, tissues were post-

fixed in osmium tetroxide, embedded in Epon Araldite and stained with lead citrate and uranyl acetate. Activity measurements for testicular and hepatic β-glucuronidase, sorbitol dehydrogenase, acid phosphatase, and testicular and λ-glutamyl transpeptidase were performed as previously described (Chapin et al., 1982).

RESULTS

Data from the hormonal assays (Table 1) indicate that LH, FSH, and testosterone levels were unchanged after 1 and 3 weeks of 2,5-HD consumption, suggesting a direct effect on the testis itself. By 6 weeks, when the testes were atrophic, plasma testosterone levels were normal, but LH and FSH were elevated, suggesting a compensatory hypersecretion in response to peripheral damage. This suggests that 2,5-HD acted directly on the testis.

Light microscopic examination of the testis after 3 weeks of 2,5-HD treatment showed no effect on the tissue. After 4 weeks, large vacuoles were present in the epithelial lining of the seminiferous tubules (Figure 1). Electron microscopy of these

TABLE 1. EFFECT OF 2,5-HD ON PLASMA LEVELS OF FSH, LH, AND TESTOSTERONE

	Week 1	Week 3	Week 6
LH, ng/ml[a]	85 ± 18	74 ± 11.3	62.8 ± 15.6
Treated-PFC,[b] ng/ml	.3 ± 20	-9.5 ± 10.4	47.8 ± 12.7*
FSH ng/ml[a]	205.0 ± 36.4	220 ± 266	258 ± 20.9
Treated-PFC,[b] ng/ml	-21.6 ± 64.4	-18.3 ± 49.2	280 ± 30.5*
Testosterone ng/100 mls[a]	406 ± 144.3	377.5 ± 114.3	616.6 ± 57.0
Treated-PFC,[b] ng/100 mls	17.5 ± 45.8	-45.3 ± 124.6	26.5 ± 106.0

Effect of 2,5-HD on plasma levels of FSH, LH, and Testosterone. Gonadotropin and testosterone data for rats fed 2,5-HD, their pair-fed controls (PFC), and ad libitum controls. (a) Mean ± SE hormone concentrations in ad libitum (N = 6); (b) The mean ± SE difference between treated and pair-fed rats. For all cells, the pair-fed hormone levels were not different from the ad libitum values; (*) Significant difference between treated and pair-fed control. (Reprinted with permission from Tox. and Appl. Pharm., 62:262-272, 1982)

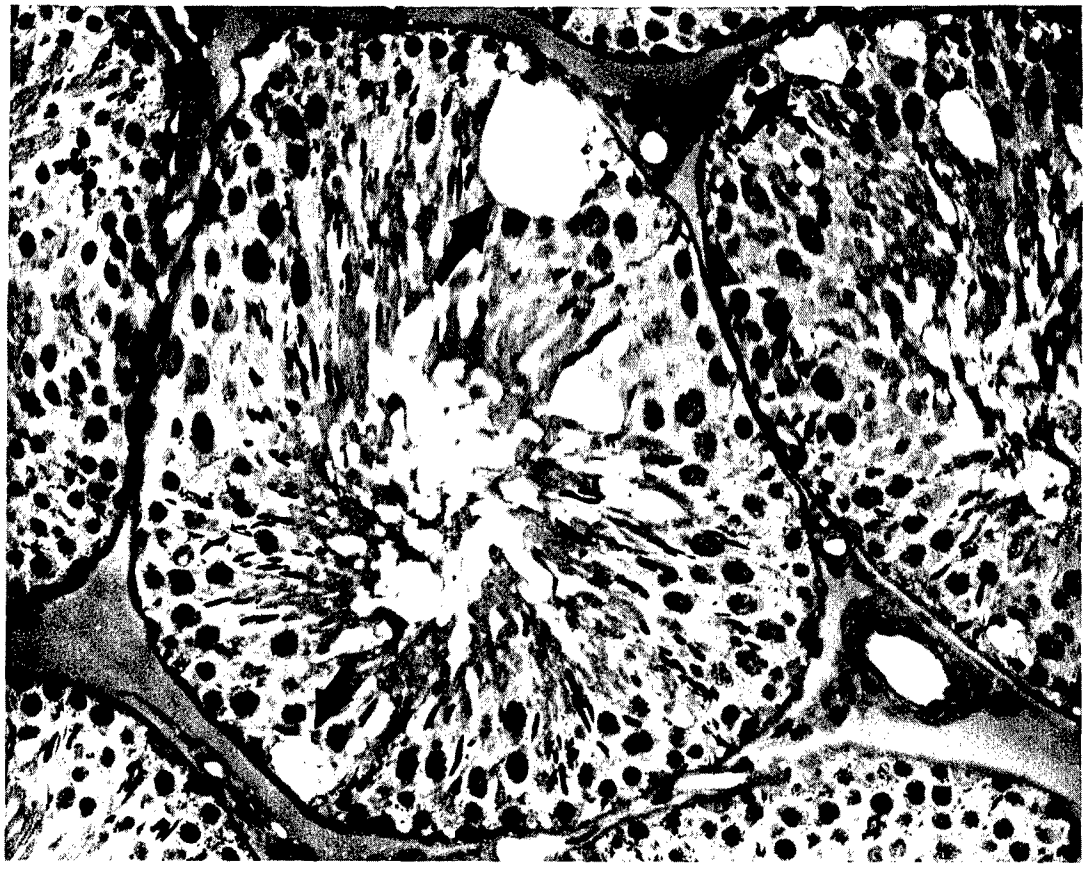

Figure 1. Seminiferous tubule from rat treated with 1% 2,5-HD for four weeks. Note large, basally located vacuoles (arrow). Stage XII. Magnified 340x. (Reprinted with permission from Tox. and Appl. Pharm., 62:262-272, 1982.)

Electron microscopy of these tissues demonstrated that the vacuoles were swellings of the smooth endoplasmic reticulum (SER) of the Sertoli cells (Figures 2 and 3). As the lesion became more severe, these vacuoles enlarged, often displacing spermatogonia and/or spermatids (Figure 4). Subsequently, early stage spermatids showed chromatin margination, often in the presence of cell exfoliation and general tubular degeneration (Figure 5). After further treatment, the spermatids fused to form the multinucleated giant cells characteristic of late-stage testicular degeneration (Figures 6 and 7). Atrophic testes, seen after 6 weeks of 2, 5-HD consumption, were composed of tubules with only Sertoli cell processes and nuclei, and spermatogonia.

Biochemical assays confirmed that Sertoli cells are a target of 2,5-HD in the testis. β-Glucuronidase is an enzyme found in the lysosomes and, to a lesser degree, in the SER of Sertoli cells; with the exception of occasional spermatogonia, β-glucuronidase is found in no other cell type in the testis (Males & Turkington, 1971). Another enzyme, λ-glutamyl transpeptidase

(GGT), is also localized primarily in Sertoli cells (Hodgen and Sherins, 1973). Acid phosphatase is found in lysosomes of many cell types in the testis (Barham et al., 1976), and can serve as a general lysosomal marker. Sorbitol dehydrogenase has been localized in developing germ cells and appears first in the pachytene spermatocytes (Mills and Means, 1972).

Treatment with 2,5-HD for just one week reduced testicular β-glucuronidase activity by 45% (Figure 8). After 3 weeks of treatment, this change was magnified, and the activity of the other Sertoli cell enzyme, GGT, was also reduced. These changes occurred in the absence of any marked change in cell number and without a concomitant change in acid phosphatase activity, suggesting that the effect on β-glucuronidase was not due to a general effect on testicular lysosomes. The liver, which is not affected histologically by 2,5-HD, evidenced a different profile of enzyme activity changes (Figure 9). There was a consistent, relatively small, decrease in the activities of both lysosomal

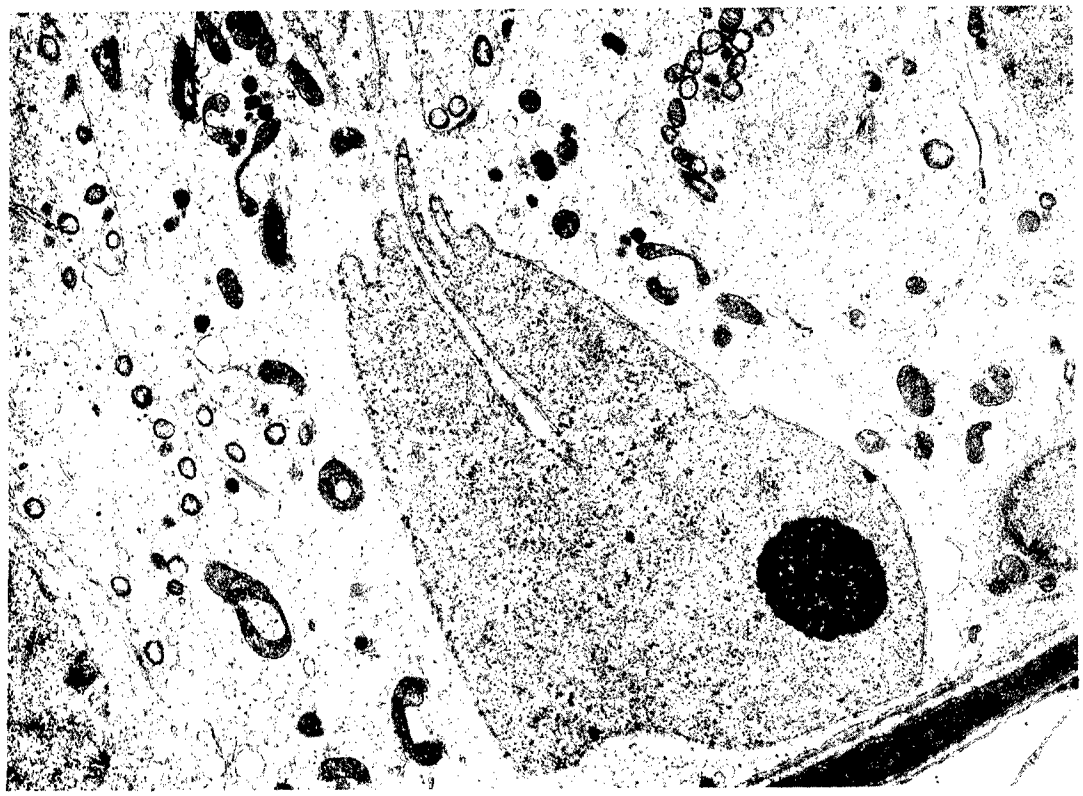

Figure 2. Sertoli cell nucleus and cytoplasm from control rat. Magnified 6290x. (Reprinted with permission from Tox. and Appl. Pharm., 62:262-272, 1982 and Experimental and Molecular Pathology (In Press) entitled "The morphogenesis of testicular degeneration induced in rats by orally administered 2,4-hexanedione" by Chapin, Morgan, and Buss, March or April 1983.)

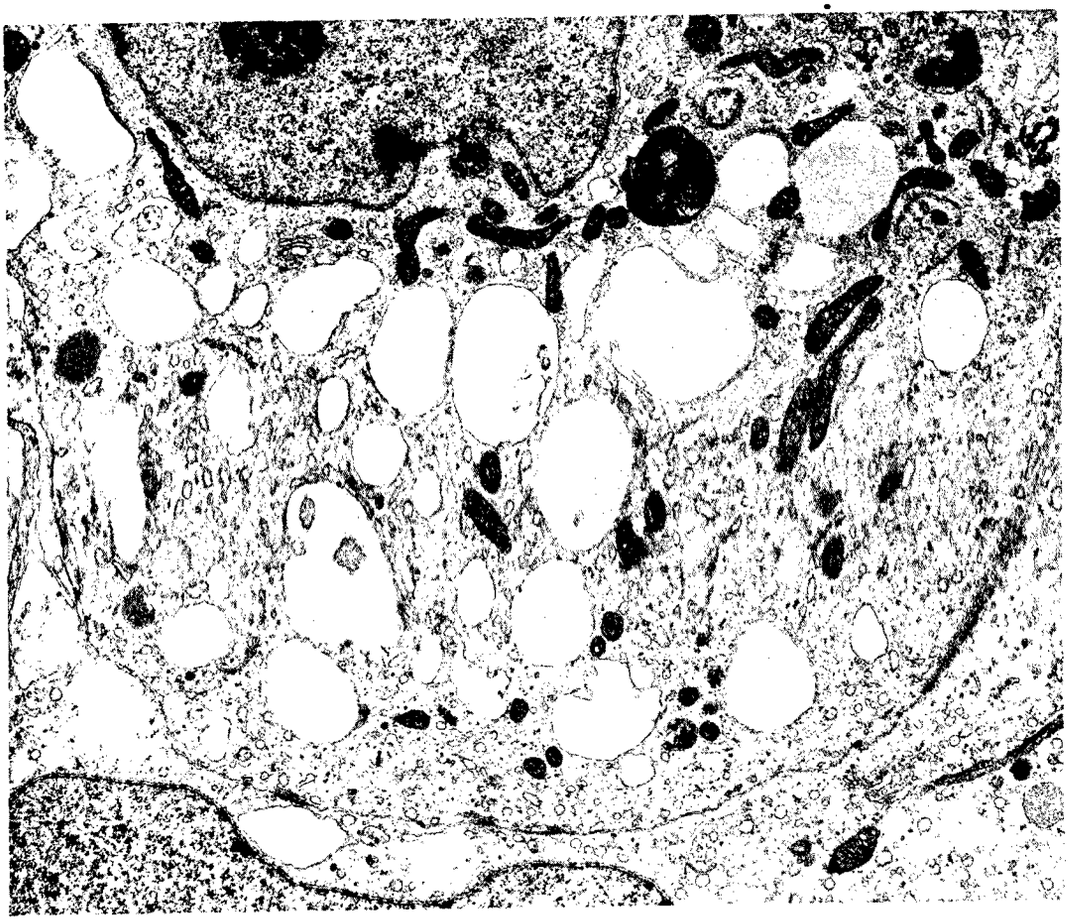

Figure 3. Perinuclear area of a Sertoli cell from a rat treated with 1% 2,5-HD for four weeks, with enlarged SER cisternae. Magnified 8800x. (Reprinted with permission from Tox. and Appl. Pharm., 62:262-272, 1982.)

enzymes, suggesting a general effect on that organelle in the liver, unlike the testis. The testicular enzymic changes seen after 6 weeks of 2,5-HD consumption may be due primarily to the loss of germ cells. At this time, there is a decrease in testis weight by 50 to 70%, with a concomitant decrease in protein content. Thus, the specific activity of enzymes in the remaining cells rises due to this protein loss. Another model of the atrophic testis, the hemi-cryptorchid rat, displays an enzyme profile similar to that seen after 6 weeks of 2,5-HD consumption (Figure 10), suggesting that the changes seen at this time are largely a result of cell loss, although one cannot tell from the data if a chemical effect is present at the six week point.

DISCUSSION

The first experiments were performed to determine if 2,5-HD acts on central hormonal control systems (brain and/or pituitary) or directly on the testis itself. If the actions are primarily central, one would expect to see decreased plasma levels of LH and

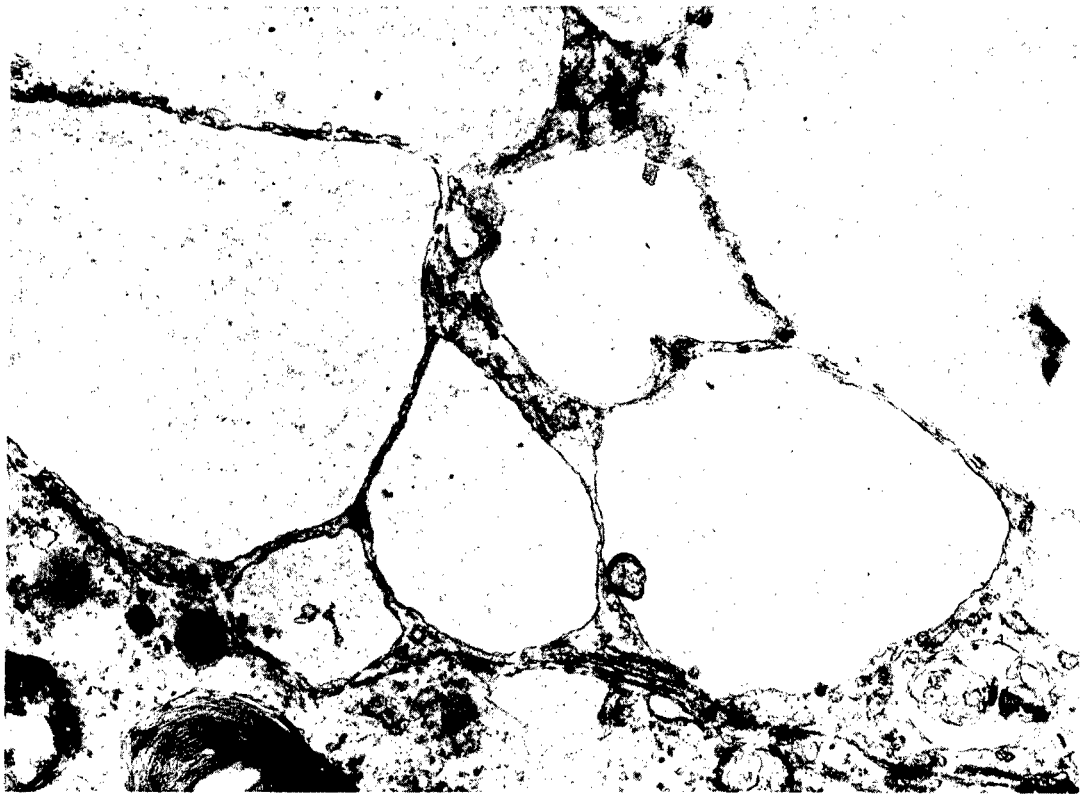

Figure 4. Cytoplasm of a Sertoli cell from a rat treated with 1% 2,5-HD for five weeks to demonstrate more severe vacuolation, with floccular material in the membrane bound spaces. Magnified 13,750x.

FSH, hormones which are responsible for the normal trophic stimulation of the gonads. However, if 2,5-HD were acting directly on the testis and not affecting the brain/pituitary, then one might expect to see testicular atrophy prior to any change in hormone levels. The above studies demonstrate that 2,5-HD, which is a peripheral neurotoxicant and causes pathologic changes in feline hypothalamic optic tracts (Spencer et al., 1980), did not produce testicular atrophy by a primary effect on the central nervous system. The only changes in circulating gonadotropin levels were observed after frank testicular toxicity was measured by other endpoints. In additional studies (data not presented) where the pituitary response to exogenous gonadotropin releasing hormone (GnRH) was compared in treated and control rats, the response to GnRH stimulation was not impaired (Chapin et al., 1982). These data point to a direct gonadal effect.

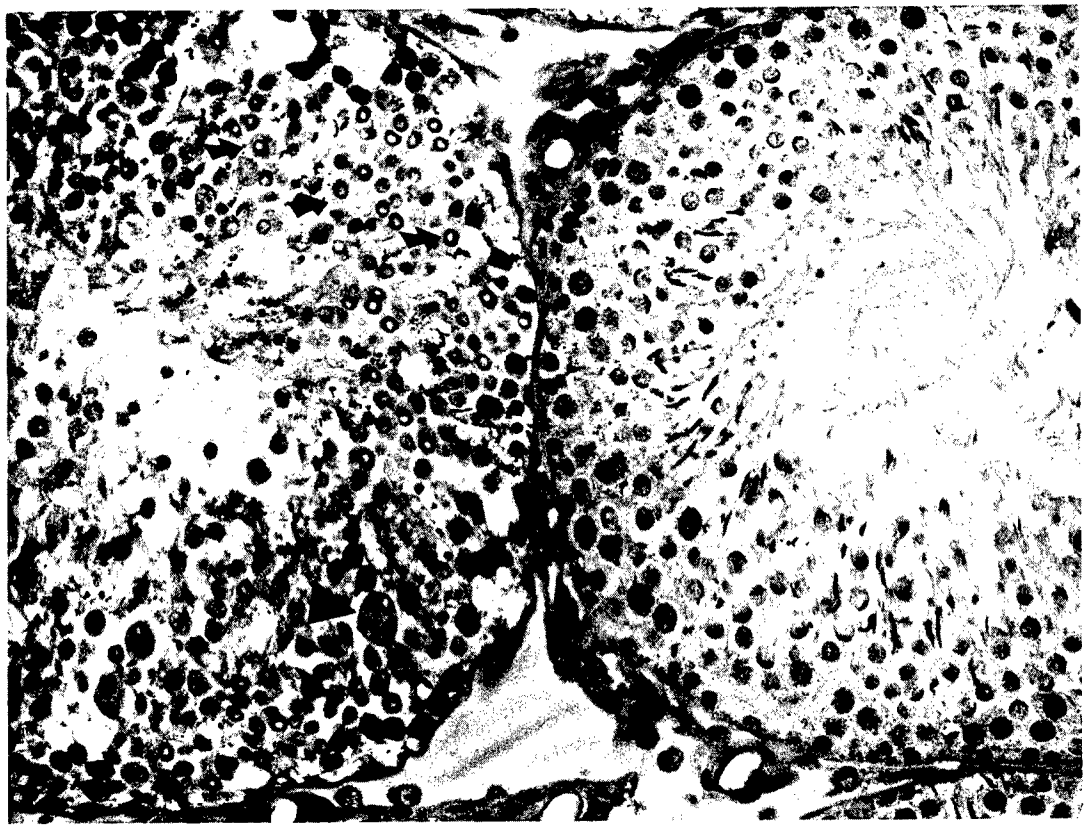

Figure 5. Seminiferous tubules from rats treated with 1% 2,5-HD for five weeks. Note karolytic spermatid nuclei (small arrows) and small multinucleated giant cells (large arrowhead) in the disorganized tubule on the left; compare with normal-appearing tubule on the right. Magnified 400x. (Reprinted with permission from Experimental and Molecular Pathology (In Press) entitled "The morphogenesis of testicular degeneration induced in rats by orally administered 2,4-hexanedione" by Chapin, Morgan, and Buss, March or April 1983.)

The Sertoli cell was the first cell type in which we observed any effect by biochemical and morphologic indices. It is noteworthy that the enzymic changes appeared after one week of treatment, and were marked by 3 weeks of 2,5-HD consumption, yet the morphologic appearance of the tissue was normal after 3 weeks, highlighting the different types of information obtainable from each technique. Also worth noting is the inability of hormone assays to predict testicular toxicity. In this study, hormonal changes appear to be reacting to the injury, rather than preceding it.

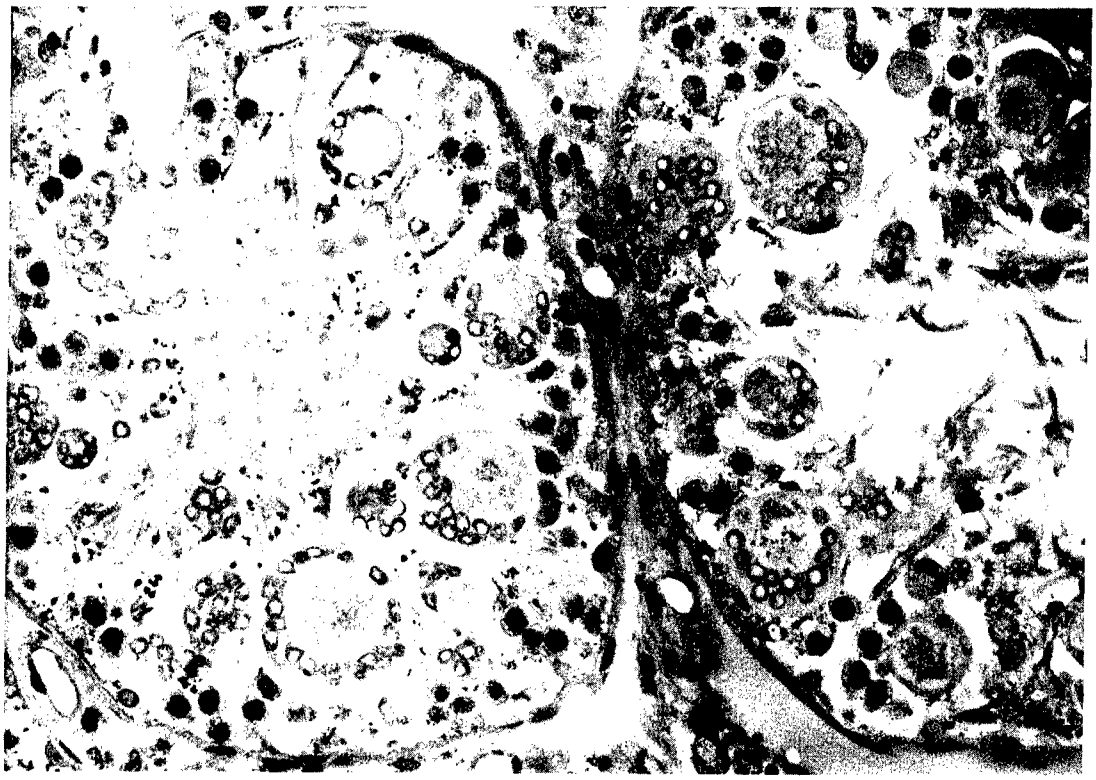

Figure 6. Seminiferous tubules from rats treated with 1% 2,5-HD for five weeks. Note the presence of numerous giant cells with crescentically arranged karolytic spermatid nuclei and a central area of floccular PAS-positive material. Magnified 400x. (Reprinted with permission from Experimental and Molecular Pathology (In Press) entitled "The morphogenesis of testicular degeneration induced in rats by orally administered 2,4-hexanedione" by Chapin, Morgan, and Buss, March or April 1983.)

Vacuoles in Sertoli cells are produced by a number of other, dissimilar compounds, and have appeared as the initial lesion after treatment with nitrofurazone, the alkylating agent Trenimon®, and pipecoline methylhydroxyindane (Uematsu, 1966; Kierszenbaum, 1970; Hausler & Hödel, 1979). Sertoli vacuolation has also been reported after induction of experimental allergic orchitis (kirszenbaum and Mancini, 1973), and concomitant with germ cell damage after treatment with AF 1312/TS (DeMartino et al., 1975). All of these authors reported that the vacuoles appeared in the SER of the Sertoli cells. The diverse nature of the treatments suggests that this vacuolation may represent a relatively nonspecific response to insult.

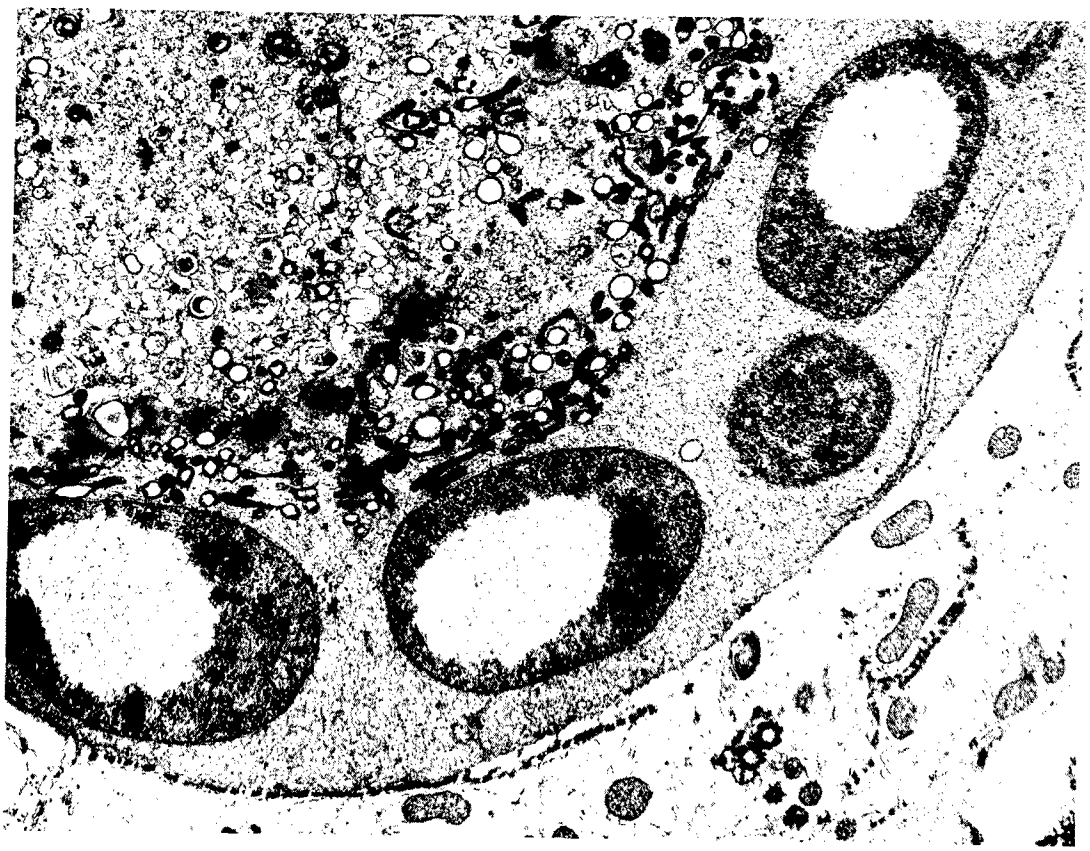

Figure 7. Electron micrograph of giant cell demonstrating membranous profiles in addition to nuclei exhibiting clumped chromatin masses and a distinct electronlucent central area. Magnified 3200x.

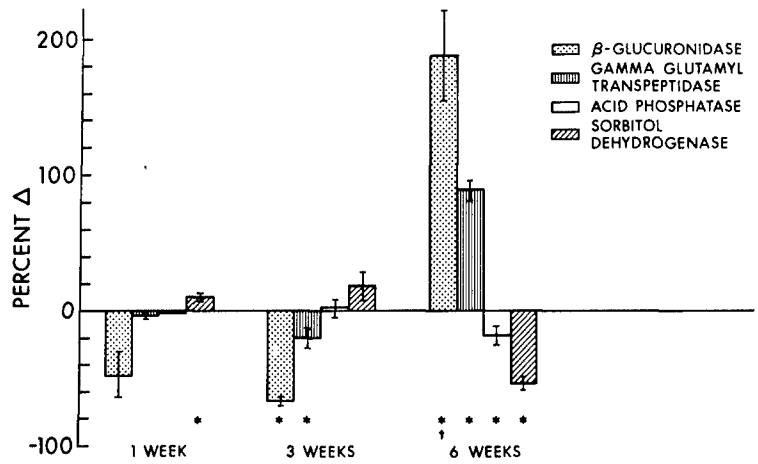

Figure 8. Effects of 2,5-HD consumption for 1,3, and 6 weeks on testicular enzyme activities. Ordinate is percentage change from pair-fed control. *Significant difference, $p < 0.05$. †Pair-fed less than ad libitum $p < 0.05$. (Reprinted with permission from Experimental and Molecular Pathology (In Press) entitled "The morphogenesis of testicular degeneration induced in rats by orally administered 2, 4-hexanedione" by Chapin, Morgan, and Buss, March or April 1983.)

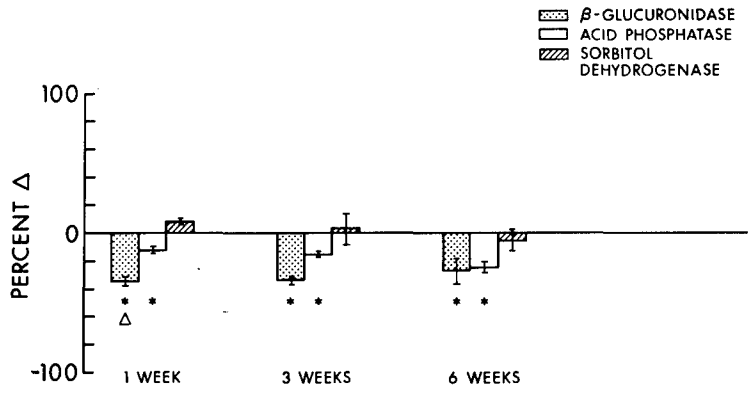

Figure 9. Effects of 2,5-HD consumption for 1,3, and 6 weeks on hepatic enzyme activities. Ordinate is percentage change from pair-fed controls. *Significant differences, $p < 0.05$. ΔPair-fed greater than ad libitum control, $p < 0.05$. (Reprinted with permission from Experimental and Molecular Pathology (In Press) entitled "The morphogenesis of testicular degeneration induced in rats by orally administered 2,4-hexanedione" by Chapin, Morgan, and Buss, March or April 1983.)

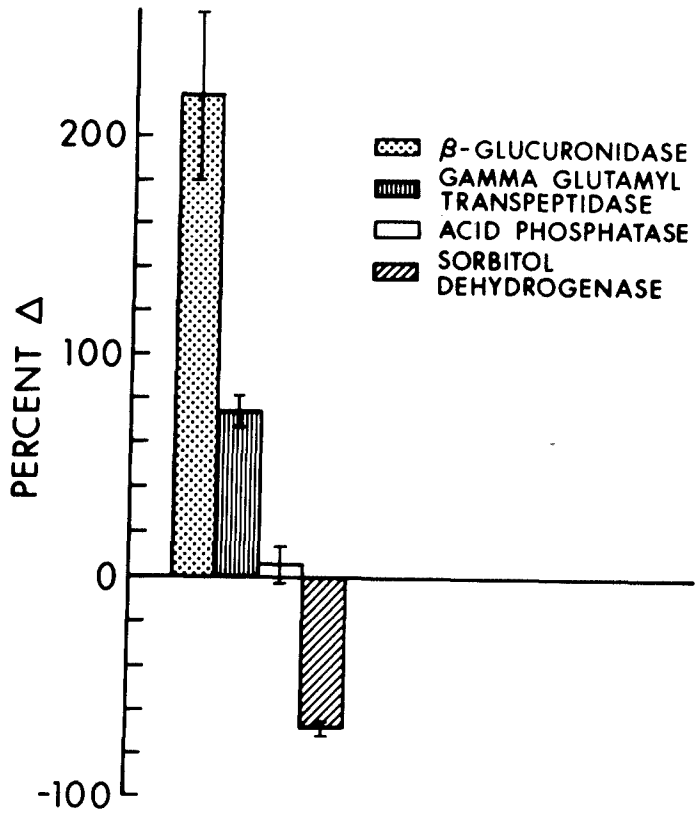

Figure 10. Enzyme activities in the cryptorchid testis. Ordinate is percentage change from scrotal testis. *Significant difference, $p < 0.05$.

The biochemical mechanisms responsible for these effects are currently unknown. The nervous system and testis share a dependence on glycolysis as a primary source of energy, and 2,5-HD produces toxic effects in both tissues. It is tempting to speculate that an impairment of glycolysis plays an important role in the development of this lesion in both tissues. However, data are not yet available to support such speculation, and investigation is continuing into the mechanism by which 2,5-HD causes testicular atrophy.

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DR. MAUDERLEY (Inhalation Toxicology Research Institute): I have a question for Dr. Springer. I missed hearing the inhalation exposure pattern for the animals to solvent refined coal in terms of hours per day, days per week. Would you repeat that, please?

DR. SPRINGER (Battelle Pacific Northwest Laboratories): The animals were exposed six hours per day, five days per week for 13 weeks.

DR. MAUDERLEY: During the daytime, I presume.

DR. SPRINGER: Right, yes.

DR. MAUDERLEY: I had one other question related to that same study, on the data for LDH. There was a difference in the LDH values at 30 and 90 days. You pointed out an increase in LDH values in the medium level exposure at 30 days and a decrease in the high level exposure at 90 days but what was most remarkable about those data was that the LDH values in control, low, and medium exposure animals were all markedly elevated at 90 days compared to 30 days. Is this an age effect, or is the reason for it known?

DR. SPRINGER: We really have an explanation for that change. I guess I just can't say much about it.

DR. MAUDERLEY: If the high level exposure group had also been elevated at 90 days, I would have considered it fairly normal. I have a question, if I could continue for Mr. Gaworski. I was looking at the data you were talking about in the petroleum versus the shale derived diesel fuel marine exposed animals and you pointed out a leukopenia. It looked to me, however, that in both of the slides only one of all the values listed was different. It was a leukocytosis in, I believe, the controls for the petroleum derived exposed animals. If it hadn't been for that one value, then you wouldn't have had anything you could call a leukopenia. Could you clarify that point?

MR. GAWORSKI (University of California, Irvine): That's correct. And I really don't know quite how to interpret that finding because if you examine historical control values that we've measured in our laboratory, that control group is higher than we would expect. And as you pointed out, if you take the values for the shale derived or the petroleum exposed groups and compare with the second set of controls for the shale materials, it fits right into a normal value. That's one of the reasons I didn't stress it at the end of the paper. I think the difference in white blood count is probably an artifact related to that control group more than a result of exposure.

DR. MAUDERLEY: It seemed as if you would have run a comparison between those two control groups; then the one would have probably been significantly elevated.

MR. GAWORSKI: I think it probably would have.

DR. MAUDERLEY: So it may not be correct to describe the difference as leukopenia.

MR. GAWORSKI: Dr. Springer also mentioned that he had found leukopenia as an effect in his study.

DR. MAUDERLEY: The third thing I would like to ask is a general question. We saw toxicity data for fuels and propellants that were very interesting, but I didn't catch any reference to concentrations that people are being exposed to or are predicted to be exposed to. How do these studies relate to human exposure levels either measured or expected?

MR. GAWORSKI (University of California, Irvine): We based our concentration models on a number of physical constraints, both of the generation system and the type of exposure that we conducted. We think they're realistic in that the concentrations we used should be representative of what a person would be exposed to in a real-life situation. And also, as I mentioned in the beginning, we wanted to get a worst-case type exposure so it actually may be a little bit higher than an individual might be exposed to.

DR. MAUDERLEY: I understand. I wouldn't expect the studies to be done at the exact human exposure levels but I was just curious to know if anyone had collected any data on human exposure levels. Thank you.

COL INMAN (Air Force Aerospace Medical Research Laboratory): I do have an answer to that question for JP-10. This material is just now going out to the field so there has not been much information gathered but there have been a couple of incidents and most of the concentrations you see in the workplace where some of this material is spilled and left around for a while is less than 2 parts per million. Pretty small amounts. But it's interesting that people get upset about it because it is highly odoriferous and they don't like it.

DR. MacEWEN (University of California, Irvine): I'd like to respond to your question with a little further information. Our original concept in working with these fuels was that we could not generate a human exposure with the entire fuel, so what we did was to vaporize that portion that would come off if we had a spill at a temperature up to 140°F, which would be about the maximum temperature of a spill on a runway or on the skin of the aircraft in Guam or some place like that. That temperature was selected to represent a maximum and there is some fractionation of the fuel in

doing this. We don't get the higher molecular weight aromatics in this fraction, as you may have noticed from the gas chromatograms of the two fuels.

DR. SLONIM (Air Force Aerospace Medical Research Laboratory): Dr. Springer, I was really struck by the fact that a number of your parameters in the mid-treatment group showed an effect at 30 days which disappeared in 90 days. Did you mention why that was so? For example, lactate dehydrogenase and several other measurements that you showed were significantly different at 30 days but that effect disappeared in 90 days.

DR. SPRINGER (Battelle Pacific Northwest Laboratories): I'm not sure we have a good explanation for that. It could possibly be due to some kind of accumulated effect or some other mechanism. I don't know that we can really say very much about it. Do you have some thoughts you'd like to offer?

DR. SLONIM: I was hoping you would! It looks like it may be a reversible process.

DR. SPRINGER: Yes, it could be that it's a reversible effect or at least partially reversible.

MR. VERNOT (University of California, Irvine): I'd like to ask a question of Dr. Inman. Did you ever look at the distribution of the radioactive material in female rats?

COL INMAN (Air Force Aerospace Medical Research Laboratory): No, I haven't. That study is scheduled to be done but has not been initiated.

MR. VERNOT: My question has to do with the hypothesis about the reason for the toxicity of the material. Col Bruner hypothesized that what might be happening was that the regulation of a hormonally controlled protein might be upset in some way to increase the concentration of the protein in kidney and that might be the direct cause of the toxicity. Your finding was that the JP-10 itself or its metabolites were highly concentrated in the kidney indicating that they might have a direct effect on the organ itself.

COL INMAN: That's possible. I didn't mention it, but the metabolite that I found in the kidney had the ketone group on the number five carbon and we have not found this anywhere else in the animal. All the other metabolites that we find in the intestine and in the urine are monohydroxy compounds. We need to look at this difference a little closer. That's why I didn't discuss it today.

MR. VERNOT: Thank you.

DR. REESAL (University of California, Irvine): My question is directed to Col Bruner. Like many pathologists, I have difficulty

sometimes in knowing what to call circumscribed clear cell proliferative lesions of the kidney. The difficulty we have is in knowing whether to call such lesions renal adenomas or renal cell carcinomas, and often the occurrence of metastases can be helpful in making this distinction. I wonder whether you observed metastases with these lesions and if you did not, what criteria did you use in making this distinction?

LT COL BRUNER (Air Force Aerospace Medical Research Laboratory): None of the tumors that we saw were of the clear cell variety; however, I think that some other investigators have seen what you're talking about. The best answer would be that you just have to go on morphologic features alone. If these circumscribed clear cell lesions you're talking about are tumors or restricted to a small segment of the tubule and they're expansible, non-invasive, with no metastasis, you'd probably have to call them adenomas. However, if you can determine there is metastasis or aggressive infiltration or invasion of adjacent tissue, or have a high mitotic index you'd have to go with a carcinoma diagnosis. That's the best answer I could give you.

DR. REESAL: Did you ever find carcinomas then? Invasive metastatic lesions?

LT COL BRUNER: None had metastasized. Based upon their size, mitotic index, and local invasiveness, the tumors we saw were judged to be carcinomas. And I'd like to make a point. When I'm evaluating a toxicity study and I find a tumor, I don't think it's really important if it's an adenoma or a carcinoma. In my opinion, they're both neoplasia and they're bad.

DR. REESAL: Thank you.

DR. WHITTENBERGER (University of California, Irvine Southern Occupational Health Center): I had a question for Dr. Chapin. You showed that the changes in FSH, LH, and testosterone did not appear until the six weeks observation after treatment with hexanedione and you indicated that those changes weren't very helpful in terms of describing the mechanism of effects. I'm curious since you've showed effects on Sertoli cells and sperm cells. What is the mechanism for the changes in LSH, FSH, LH and what were the mechanisms that led to the changes?

DR. CHAPIN (National Institute of Environmental Health Sciences): We didn't do studies to address that directly. The release of both LH and FSH are stimulated by a single compound. That is, RH from the hypothalamus stimulates the release of both LH and FSH. That makes it kind of messy because they each apparently have a different feedback partner; that is, LH has testosterone and FSH may or may not have inhibin. If you, for example, kill the Leydig cells, you will see a rise in both LH and FSH in plasma although it's responding only to the loss of testosterone. In our study, I

think the brain was responding to a loss of inhibin and it was boosting FSH. The LH just kind of piggy-backed along suggesting that the brain is not significantly affected by the compound and the response mechanisms are still intact. Did that answer your question?

DR. WHITTENBERGER: Yes.

DR. WHITTENBERGER (University of California, Irvine Southern Occupational Health Center): You mentioned the preservation of the stem cells and suggested there might be recovery and that you had further experiments underway. Are those further experiments far enough along for you to give any indication of the probable result?

DR. CHAPIN (National Institute of Environmental Health Sciences): Generally, to fully assess recovery, one needs to wait for two cycles of the seminiferous epithelium and a cycle is seven weeks long. With the six-week dosing period plus 28 weeks of waiting, we're just now in the middle of that study and the rats are still alive. We haven't done any pathology yet.

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