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IMMUNOLOGIC ASPECTS OF BERYLLIUM DISEASE

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UNIVERSITY OF CINCINNATI COLLEGE OF MEDICINE

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13. ABSTRACT

Report reviews reasons that chronic beryllium disease (CBD) has been thought to have immunologic pathogenesis. It suggests how CBD fits the immunologic disease concept. CBD humoral factors study suggests antibody may be directed at gamma A, M, and G. Humoral transport of beryllium remains unclear. Immunoelectrophoresis and autoradiography of rat serum after Beryllium injections did not show any association of radioactivity with serum proteins. Culturing techniques for human, guinea pig and rabbit lymphocytes are described. Rabbit and guinea pig sensitizing methods are discussed. Rabbit lymphocytes are best for in vitro culturing but, as yet, have not been stimulated by beryllium. This report describes an instance of successful passive transfer of beryllium hypersensitivity by local transfer of cells; systemic transfer is less convincing. Pursuit of culture techniques with emphasis on in vitro diagnostic testing is recommended.

Key Words: Chronic beryllium disease
Berylliosis
Autoimmunity
Immediate hypersensitivity
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Delay hypersensitivity
Cellular immunity
Patch test
Skin test
Passive transfer
Immunoelectrophoresis
Fluorescent antibody technique
Latex fixation test
Sheep cell agglutination test
Lymphocyte culture

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The experiments reported herein were conducted according to the "Guide for Laboratory Animal Facilities and Care," 1965 prepared by the Committee on the Guide for Laboratory Animal Resources, National Academy of Sciences – National Research Council.

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SUMMARY

In this report we have reviewed the reasons that chronic beryllium disease has been thought to have immunologic pathogenesis.

Humoral factors in chronic beryllium disease were sought in human patients and in sera from monkeys exposed experimentally to beryllium. A significant number of the human sera showed the abnormal presence of factors giving agglutination of sensitized latex particles which would, theoretically, indicate an antibody to gamma globulin. This suggests that the elevated gamma globulin seen in active chronic beryllium disease may be directed at something specific. It is doubtful, however, that it would all be directed at gamma globulin. This study has also shown that chronic beryllium disease patients can have elevations of the gamma A and gamma M immunoglobulins as well as the gamma G type. No alterations were discovered in Beta 1A globulin or in more than twenty other serum proteins that would be of diagnostic significance. No antinuclear or anticytoplasmic antibodies were detected by the fluorescent antibody methods.

Humoral transport of beryllium remains unclear. We did not find evidence of beryllium binding to serum proteins either by immunoelectrophoresis (IEPA) with laser spectrographic analysis or IEPA with radiographic methods. One possible mode of humoral transport is as the inorganic compounds beryllium phosphatase and beryllium hydroxide.

Since beryllium is able to bind to skin alkaline phosphatase, a portion may be transported in bound form with serum alkaline phosphatase. Two cellular mechanisms may also contribute to beryllium transport. First, particulate beryllium may be transported as phagocytized substances, and secondly, soluble particles of beryllium may bind to intracellular DNA and RNA.

Beryllium antigen may be the result of one of the reactions noted above where beryllium binds with DNA, RNA or alkaline phosphatase. Interactions of the tissues with beryllium with alteration not inducing beryllium binding is also a strong possibility. In some of our experiments we attempted to prepare a useful beryllium antigen without attempting to characterize its chemical nature. The extraction attempts did not result in any preparations that were able to stimulate lymphocyte cultures. However, some new ideas have come out of this work which are theoretically very promising.

Techniques for culturing lymphocytes from humans, guinea pigs and rabbits have been described and some difficulties and pitfalls discussed.

Methods for sensitizing animals have been discussed and it was shown that rabbits are much more easily sensitized by beryllium than guinea pigs and would probably be the best experimental subjects for such studies, especially where in vitro lymphocyte culturing is involved.

Some experiments have shown that beryllium hypersensitivity could be transferred passively with cells using the local transfer method. An experiment using the systemic transfer method was less convincing and further replications of these studies are needed.

Since beryllium could not be shown to bind to proteins, we decided to form a "chromium antigen" as a model system and attempt to extract that from tissues. Toxic factors were encountered in this extract and no conclusions as to the presence of the chromium antigen could be drawn.

Lymphocyte cultures were used for some studies with humans having chronic beryllium disease but neither beryllium solutions or beryllium-containing tissue extracts were found to be stimulating. Difficulties with guinea pig and rabbit culture techniques were just being solved at the conclusion of this work; in fact, success with guinea pig cultures was realized only after the termination date. These culture techniques can now be pursued with emphasis on the development of an invitro diagnostic test.

FOREWORD

This report was prepared by the Kettering Laboratory of the Department of Environmental Health, University of Cincinnati College of Medicine, Cincinnati, Ohio 45219. It covers research performed between 1 January 1967 and 30 June 1969 under USAF Contract F33615-68-C-1320 initiated by the Aerospace Medical Research Laboratory, Wright-Patterson Air Force Base, Ohio. Sheldon A. London, PhD, Chemical Hazards Branch, Toxic Hazards Division was the contract monitor for the Aerospace Medical Research Laboratory. The research was performed under Project 6302,"Toxic Hazards of Propellant and Materials",Task 630205, "Toxic Hazards Evaluation of Aerospace R&D Operations", and is a continuation of projects started under USAF Contract AF33(657)11036. The author acknowledges the invaluable technical assistance of Mrs. Janet P. Raines. Lloyd B. Tepper,MD provided encouragement and liaison with the physicians in charge of beryllium disease patients and assisted in the collection of blood specimens. E.V. Perrin,MD, and A.J. McAdams,MD, provided the fluorescent antibody studies. E.V. Hess,MD, provided the Latex Fixation Tests and the Sheep Cell Agglutination Tests. The following physicians either sent or allowed us to collect serum specimens; Harriet L. Hardy,MD, Joseph M. DeNardi,MD, John Cunney,MD, Harold L. Israel,MD, H.S. Van Ordstrand,MD, Warren D.Bobb,MD, and Clyde Woodworth,MD. Sheldon A. London,PhD, provided the monkey serum specimens.

This technical report has been viewed and approved.

C.L. HOLT, Colonel, USAF, MC
Commander
Aerospace Medical Research Laboratory

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SECTION I

INTRODUCTION

There are many industrial, military and research interests in the metal beryllium. It is extremely hard and corrosion resistant, has a high melting point, is light in weight and is almost transparent to x-rays. It has a prominent place in atomic energy utilization, and, in the finely divided state, is an extremely powerful solid propellant for rockets. The oxide of beryllium is widely used as electrical insulation with excellent heat-dissipating ability. The hardness of this oxide is such that plates backed with fiberglass and worn as body armor can stop 30 calibre armor-piercing bullets, and the toxicity of such oxides when formed at high temperatures is reported to be much reduced (ref.1).

This laboratory has been interested in the industrial hygiene aspects of beryllium and its compounds for many years. Although strict air control programs have led to great reduction in the incidence of beryllium disease we believe that an understanding of the disease mechanism in human beings will lead to even more satisfactory hygiene measures by emphasizing areas of high risk.

An industrial hygiene study at Kettering Laboratory (ref.2) showed that in certain operations, such as the production of copper beryllium alloys, the threshold limit values in actual practice have been exceeded several fold. At the industrial site investigated, no cases of chronic beryllium disease have been reported in this area. In another area which is in a separate building, there is allegedly excellent conformity to the threshold limit values, yet this area has yielded cases of chronic beryllium disease. It is questioned, therefore, whether there could be a qualitative difference in the form of beryllium resulting from the two operations and whether the one is more toxic or immunogenic than the other.

Clinical disease was first reported in the beryllium industry in 1933. This and other studies reviewed by Tepper et al.(ref.3) failed to distinguish between the acute form of beryllium disease and what has come to be known as chronic beryllium disease. In 1946 Hardy and Tabershaw (ref.4) distinguished between a short self-limited disease seen in the beryllium production industry and a progressive disease of delayed onset seen in beryllium-use industries. Subsequent follow-up in the production industries has revealed that cases of the progressive disease can appear there also (ref.5). An extensive epidemiologic study of beryllium intoxication cases was reported in 1950 by Sterner and Eisenbud

(ref.6). They reviewed over 100 cases of chronic beryllium disease and concluded in the light of immunologic theories, the work of Curtis with the patch test (ref.7) and their own accumulated data, that chronic beryllium disease could be of an allergic or immunologic etiology. A current immunologic working concept of this disease would differ in some details from their hypothesis, but this in no way mitigates the insight manifested by their original exposition. Fifteen years later, Van Ordstrand's experiences andclinical observations (ref.8) have allowed him to feel confident in the immunological concept; however, Hardy (ref.9) cautioned against using the immunological concept prematurely as fact to the possible detriment of industrial workers and urged that continued studies be done.

A current working concept of immunological disease in general requires that some substance be recognized by the body's immune system as a foreign entity or "antigenic" substance. This antigenic substance, usually a large molecule, may arrive from the environment as a complete antigen easily recognized as foreign, or it may have to bind with or otherwise alter a body substance in such a way that the immune system reacts to it as if it were foreign. Once recognized or believed to be foreign, the body's lymphoid tissue will produce an immunologic response which is manifested by the development of germinal centers in the lymph nodes where cellular proliferation takes place. Immunologically competent, small mononuclear cells that are not "committed" to any particular antibody formation become committed to this antigen and form blast cells and begin to divide in response to this foreign substance and become committed to this antigen. These cells migrate from the lymphoid tissues into the blood and other tissues and then circulate back through the lymphoid tissues. They will recycle until they encounter the antigen. This results in an accumulation of these lymphocytes in areas near the antigen and the release of toxins that cause tissue damage. This type of response involving cells alone and no detectable antibodies is called cellular hypersensitivity, bacterial hypersensitivity, or delayed hypersensitivity (DHS). These cells are not the antibody-producing cells of the body, and recent experiments (ref.10) indicate that they must interact with bone marrow cells before significant antibody production can occur. Presumably they pass their recognition information on to some other non-committed cell which develops into the plasma cell that produces antibodies. This type of immunologic response, where antibody is produced, is called the antibody response, humoral response, anaphylactic response, or immediate hypersensitivity response. A common accompaniment of this response is the serum complement system. This is a complex system of interacting proteins which join into the antigen-antibody reaction

and may be responsible in large part for cytotoxic damage.

In summary then, an antigen can elicit production of committed cells and/or committed antibodies, both of which are in circulation throughout the body and can react with the antigen wherever it is encountered. Various combinations of the two responses can occur; one, the other, or both of these systems may be important in the case of a specific antigen.

Comprehensive study of a suspected immunologic phenomenon should include consideration of the nature of the antigen and of both the cellular and the antibody responses. Let us consider how clinical and experimental evidence might fit chronic beryllium disease into this concept.

In chronic beryllium disease the existence of a "beryllium antigen" is not in doubt; however, the nature of the antigen is not known. The certainty of its existence lies in the fact that while men and animals do not react initially to the skin applications of beryllium solutions they do react after prior exposure (ref.7,11,12,13). In our own work with guinea pigs and rabbits, both can be sensitized by skin applications to give positive reactions to intradermally injected beryllium. In terms of the concept elaborated above, beryllium is too small in molecular weight to be a complete antigen, thus it must bind with or cause alteration or denaturation of some substance in the tissues or body fluid in order to create the antigen. This interaction could take place in skin, mucous membrane, lung or wherever beryllium is transported. In the natural human exposures and the animal studies referred to above, skin surface contact seemed to be a common denominator. Experiments performed by Voisin et al. (ref.14) using subcutaneous injections resulted in no immunologic sensitization detectable in the skin. Some speculation about the nature of the antigen can be made from studies of beryllium-binding in the epidermal portion of guinea pig skin by Belman (ref.12) indicating that beryllium binds to the protein alkaline phosphatase and to both nucleic acids, RNA and DNA. Further studies in the same work showed binding of the beryllium both to native and heat denatured bovine serum albumin. Therefore, we have some evidence that beryllium does interact with tissue constituents. Another report (ref.15) concluded that beryllium did not interact with human serum protein but was probably transported in the body fluids as beryllium phosphate and hydroxide. Our own studies concerning protein-binding using immunoelectrophoretic analyses reveal no beryllium associated with serum proteins.

Beryllium must be able to get to internal tissues if the antigen is to be created and, indeed, the work of Spencer et al.(ref.1) shows that intratracheally administered beryllium can be found in liver, kidney and spleen after several weeks.

Humoral factors should be considered for support of an immunological concept of the disease. However, without a test antigen, many specific experiments cannot be done. The skin reactions in humans and animals that confirm the existence of such an antigen are all of the DHS type, which is evidence against a specific circulating antibody to the beryllium antigen. Such a reaction would be expected to occur either after 20 minutes or perhaps at 4-6 hours. All of the skin test reactions reported by various investigators required well over 24 hours to reach their maximum intensity. It is well known, however, that many patients with chronic beryllium disease have elevations of serum gamma globulin during the active period of the disease (ref.16). If this gamma globulin is not antibody directed at beryllium antigens in the skin, then the question must be raised as to what it might be directed toward. Interactions of beryllium with tissues other than skin would likely give antibody with different specificity that would not react with skin. Since we have noted that beryllium can react with various body components and can be transported to many sites (ref.1), perhaps the antibody is directed toward some altered internal body component. There is increasing evidence that this type of mechanism can occur.

In several other clinical conditions serum antibody does indeed become directed against body tissues. These may be altered body tissues; however, after the recognition mechanism is once deceived by the alteration, antibody must be developed against the entire molecule and such antibody can then react with normal tissues. For example, systemic lupus erythematosis is a disease in the active stages of which antibodies can be detected that are directed against nuclear materials of the body cells (ref.17). Using the fluorescent antibody technique (ref.18), these can actually be demonstrated as binding to the nuclei of normal cells. In rheumatoid arthritis, antibodies called rheumatoid factors have been demonstrated that are directed against gamma globulin itself (ref.19). There are five different types of antibody molecules and in this situation, one of the types, the gamma M antibody, is directed against another, the gamma G antibody, and the combination of the two after they have reacted is called the rheumatoid factor. This factor is also found in patients with Caplan's syndrome, a form of silicosis with round nodular fibrosis of the lungs (ref.20). Caplan's syndrome may be seen in patients with or without rheumatoid arthritis and often precedes the onset of arthritis symptoms by several years. Thyroid disease is another situation where antibodies can be

directed against normal tissue (ref.21); again, the fluorescent antibody techniques demonstrate their presence.

Serum complement was mentioned before as an accompanying factor in many antigen antibody reactions. Certain kidney diseases (ref.22) show a severely decreased Beta 1A globulin (the third component of the complement system) concentration which is apparently due to its being bound in the kidney as part of an antigen antibody complement complex.

Several animal experiments have been reported where attempts to discover evidence for humoral factors have failed. Reeves and Vorwald (ref.15) looked for humoral factors by attempting to create an antigen by incubating beryllium oxide with sera. The separated sera were not antigenic as tested by skin injection for Arthus reaction or DHS, nor could they elicit systemic anaphylaxis in guinea pigs. Alekseyeva et al.(ref.23) were also unable to detect any antibody formation directed toward beryllium. In all of this work, various mixtures of beryllium and protein were tried. The failure to elicit antibody could be caused by either the beryllium antigen not being created in this in vitro reaction or because beryllium antigen does not elicit a humoral response. Our own experience has never shown positive intradermal skin test reactions prior to 24 hours, which supports the belief that even though strong DHS reactions may be present in the sensitized animals, there is no suggestion of the presence of circulating antibody. In the human disease, however, the elevation of gamma globulin during active disease strongly suggests humoral factors in that situation.

Even though we consider the existence of humoral factors in chronic beryllium disease likely, we would agree with most investigators studying chronic beryllium disease that it is mainly the delayed type of hypersensitivity (DHS) which produces the main clinical and pathological manifestations. The patch test experiments (ref.7) showed lymphocytic infiltration and a 24 hour delayed appearance typical of this reaction. The lung lesions show all the same elements, including lymphocytes, of other diseases (ref.24) now generally considered to be primarily DHS phenomena.

In animal experiments, Voisin et al.(ref.14) could not induce convincing DHS reactions to skin testing using various techniques. Alekseyeva (ref.25) concluded that he was able to produce DHS reactions and also transfer them passively with cells. He was using a lesion-scaling system with which we are not experienced and so found it difficult to evaluate his data. For example, in the transfer experiments the recipient animals had values twice the controls. The scaling system allows one

to double the numbers based upon the judgement of 0.5 mm of lesion elevation. That is, an elevation of 0.4 mm is not doubled, but 0.5 mm is. Bias, however unintentional, could enter at this point. Also, his skin testing dose gave measurable lesions that persisted up to 8 days, which is not a desirable occurrence. He also noted the occurrence of DHS type reactions to beryllium chloride after some non-specific material injections, thus raising some questions about the specificity of the other reported data. That is, however, the first effort described where the very important passive transfer experiments have been attempted and further attempts to establish this should be made.

The sensitization described by Belman (ref.11) in guinea pigs was elicited by application of the challenge to the skin of the back. It was not reported, however, whether the same site was used for challenge as was used for sensitization. If it was, then the phenomenon he reports may be an epicutaneous form of retest reaction (ref.26,27) or the prepared site reaction (ref.28). Polak et al. (ref.13) also used this method with success in inheritance studies, thus further supporting the usefulness of this method as a sensitizing as well as establishing a method for obtaining an adequate number of highly sensitizable guinea pigs for experiments. These results are open to the same questions about retest reactions. Chiappino et al.(ref.29) have described lung lesions in guinea pigs similar to those characteristic of DHS in response to endotracheal beryllium oxide. They found immunoblasts in regional lymph nodes in response to beryllium skin applications. Skin testing the animals also showed positive intradermal reactions to beryllium sulfate; however, the concentrations used for skin testing were many times higher than our experience found irritating and this result needs further clarification.

The conflicting results of the various investigators working in this field and the unclear portions of many experiments that have been reported suggest that further animal studies should be performed.

Human clinical and pathological considerations that support the immunologic etiology of chronic beryllium disease should be summarized. The first point was made by Sterner and Eisenbud (ref.6). They noted that the chronic form of beryllium disease (which they called berylliosis) did not manifest a predictable dose response. Persons exposed to large enough doses to cause acute beryllium disease did not usually progress to the chronic disease, yet other persons exposed to very small doses have developed severe, even fatal, chronic beryllium disease. Since a host difference seemed apparent, some form of allergy was considered. They quoted the work of Curtis (ref.7) which reported

positive patch tests in exposed patients while control individuals had no reaction to the same dose patch test. Many normal controls, however, 10-14 days later developed eczematoid reactions and thereafter gave typically positive patch tests. This certainly demonstrates the susceptibility of human subjects to the skin route. Skin histopathology showed early perivascular lymphocytic infiltration with lymphocytes predominating in the subsequent lesions and granuloma formation in some. They noted further the similarity of pulmonary histopathology to the pulmonary lesions of tuberculosis which is considered a classical form of delayed hypersensitivity response. Elevation of the serum gamma globulins during active periods (ref.16) of the chronic disease favors the immunologic concept as does the clinical experience that corticosteroid drugs have proved to be of considerable benefit in controlling the disease (ref.30).

Since there is good reason to believe that chronic beryllium disease is of immunologic pathogenesis, or one of the autoimmune diseases, some of the more recently developed means of studying this type of disease will be considered here.

The technique of immunoelectrophoretic analysis (ref.31) is a very powerful tool for studying proteins, while the agglutination test (ref.19), the Sheep Cell Agglutination Test (ref.32) and the fluorescent antibody technique (ref.18) are all important methods in studying autoimmune diseases.

Culturing of lymphocytes in the past few years has been the subject of much interest among immunologists and hematologists in that these cells can yield diagnostic information in certain immunologic diseases. In 1960 Nowell (ref.33), in culturing lymphocytes for other purposes, realized that phytohemagglutinin (PHA), a substance extracted from the red kidney bean, used to separate red cells from the leucocytes, was in addition acting as a mitogenic stimulant to the lymphocytes present in the subsequent cultures. In 1963 Pearmain (ref.34) hypothesized that this mitogenic stimulation was acting through the cell's immunological response mechanism. In experiments he showed that animals sensitized to tuberculoprotein had lymphocytes that responded to that protein in a manner identical to their response to PHA, though not quite so intense, while lymphocytes from control animals could not be stimulated by the protein. Since then, this phenomenon has been demonstrated for many other antigens and in several other animal species.

These methods are all theoretically applicable to the study of chronic beryllium disease. The objectives of the studies carried out here were to test patients and experimentally exposed

monkeys for humoral factors related to immunologic disease phenomena, and to adapt some of the new immunologic methods for experimental purposes to establish further the DHS character of beryllium hypersensitivity. We also anticipated that the techniques thus applied would be usable for in vitro diagnostic testing in human populations.

SECTION II

MATERIALS AND METHODS

A. Methods for Humoral Studies.

The work described in this section utilized sera from human beings with chronic beryllium disease and sera from monkeys exposed experimentally to beryllium-containing rocket fuel exhaust products. All patients with chronic beryllium disease were accidental industrial exposures. No one was experimentally sensitized for any of these investigations.

The human sera used were obtained from patients of the physicians listed in the foreword. Some were transported by mail to this laboratory while others were collected personally by the investigator. These patients were either in the category of active disease requiring steroid for control or were so-called burned out cases no longer requiring steroid therapy. There were no cases of the acute disease nor were there newly developed, untreated cases of the chronic type disease.

Animal sera were obtained from exposed Macaca mulatta monkeys. These sera were obtained from animals exposed at the Bionetics Research Laboratory at Falls Church, Virginia. The material used for these exposures was rocket exhaust products containing 97.72% BeO (Atlantic Research Corporation Sample No.24) having a median particle diameter of 1.29 microns. The animals were exposed 3 times to nominal concentrations of 4000 micrograms Be/M^3 for 30 minutes at monthly intervals. They were then sacrificed at one year post exposure intervals for pathological studies.

The examination of these sera for antitissue antibodies was performed by the Coon's Fluorescent Antibody Technique using the multi-layer or sandwich method (ref.18). The sera were placed on cut,frozen sections of human tissue. Human thyroid,skin,lung and muscle tissue were used as test tissues as will be further discussed. Known positive and negative specimens were included in each day's run. The antigamma globulin used in the human studies was also used for the monkey sera. A separate experiment showed that the human antiserum gave excellent cross-reaction and would be adequate for study of monkey serum for antitissue factors. In fact, many of the proteins of the monkey serum showed excellent cross-reaction with antihuman serum.

Latex fixation tests were done by commercially available latex slide test. Again, both human and monkey specimens were treated with reagents intended primarily for human serum, but as noted before, excellent cross-reaction occurred. The sheep

cell agglutination test (SCAT) was done by the technique described by Ziff et al.(ref.32).The latex slide test is a very sensitive screening test for the presence of rheumatoid factor in serum, but there are some false positive reactions. The SCAT is also very sensitive, but much more specific. Since it is also technically more difficult, it is done only on sera that are positive by the latex slide test.

The remaining evaluations were all done by the micro-immunoelectrophoretic analysis (IEPA) method of Scheidegger (ref.31) and an extension of that method developed in this laboratory for obtaining a better quantitation using eight different concentration combinations of antigen and antibody for each specimen studied. This technique will be referred to as the eight slide quantitation method (8SQ). Since this has not been described before, a detailed description is presented. First,however, immunoelectrophoretic analysis will be reviewed briefly.

The IEPA method provides what might be thought of as a two dimensional analysis and the 8SQ extension of this method provides a third dimension. First, the electrophoresis provides some separation of the proteins in the serum, then the antiserum is allowed to diffuse in, perpendicular to the line of electrophoresis, while each individual spot of protein enlarges by radial diffusion. Since the rate of diffusion is a rather constant and characteristic property of each individual protein, this constitutes the second dimension of the analysis with the result that each protein precipitates with its own characteristic position. A proper antigen to antibody ratio must be selected in order to achieve an equivalence point to precipitate the material of interest. Since a quantitative screening technique was desired, various ratios of antigen to antibody were tried. It was found that eight combinations would effectively span the full range of serum protein concentrations being encountered in these experiments.

The antiserum was prepared in a goat by the injection of 0.6 ml of a pool of normal human serum homogenized with 1.9 ml of normal saline and 2.5 ml of complete Freund's adjuvant. Injections of 1.25 ml each were made into four different sites at weekly intervals until good blood levels of antibody were obtained, and these were maintained by monthly injections when the animal was not being bled. The pool of normal human serum was made by collecting small samples from a blood bank so that 100 ml contained sera from 30-50 people. Later, when the monkey studies were added, the goat was given 0.3 ml of human serum and 0.3 ml of monkey serum in each booster injection.

To prepare an antiserum pool, the goat was injected weekly to achieve an anamnestic response. This was followed by bleeding 200 ml between the 5th and 7th post-injection days. The goat was immediately boosted again and the cycle was repeated. It was important to have a large pool of antiserum stored since this could act as a reference standard for a long period of time.

In the actual performance of the procedure, 25 ml of melted 2% agar (Special Noble) in veronal buffer, pH 8.2,ionic strength 0.05, was pipetted onto a glass plate 1¼"x3"x1/8" and allowed to solidify. Eight sets of wells and troughs were cut in the configuration and dimensions shown in Figure 1. Figure 2 shows the locations of the various serum and antiserum dilutions. The serum dilutions are made in small plexiglas trays (Figure 3) by placing 3 drops of 0.9% sodium chloride solution from a disposable Pasteur pipette into each of 4 holes, then with the same pipette adding 3 drops of serum to the first hole and while the pipette is in hand, full strength serum is placed in the wells of steps 4,3,2, and 1 (Figure 2). With a second pipette, this is mixed and 3 drops are placed in the second hole, the appropriate agar well is filled and the remainder of this dilution is placed back in hole number 1. With a separate pipette, the liquid in hole number two is mixed and the cycle repeated until all dilutions have been made and placed in the agar. The Pasteur pipette is held in a nearly horizontal position to minimize slight differences in orifices. The tray of dilutions may be covered with tape such as Magic Mending Tape (No.810) and the dilution stored in the refrigerator for later use in case any technical difficulties occur with the analysis. Note that the wells above and below any one trough are duplicates of the same concentration. The agar plate is then electrophoresed until the albumin spot, which can be seen as a clear area in the agar, is 11-13 mm from the well of origin. This requires approximately 60 minutes at a current flow of 85 ma. Antiserum is diluted serially with equal volumes of saline to prepare 4 working solutions: full strength ($1/2^0$), half-strength ($1/2^1$),quarter strength ($1/2^2$) and one-eighth strength ($1/2^3$) (Figure 2). After electrophoresis, these antiserum dilutions are placed in the troughs and the plates are incubated overnight at room temperature and the precipitin arcs develop.

The location of typical precipitin arc end points of normal serum are shown in acomposite drawing in Figure 4. The interpretation of theresults is accomplished by proceeding from one protein to another until all have been covered. The results for each protein are recorded as the number of the step and fraction of a step in which the sharpest, thinnest arc is located. This is the point where a particular antigen and antibody are in equivalence. One arc after another is examined up and down the

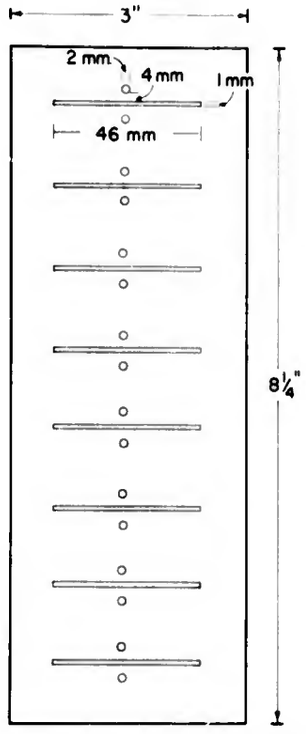

Figure 1. Configuration and dimensions of the agar mold.

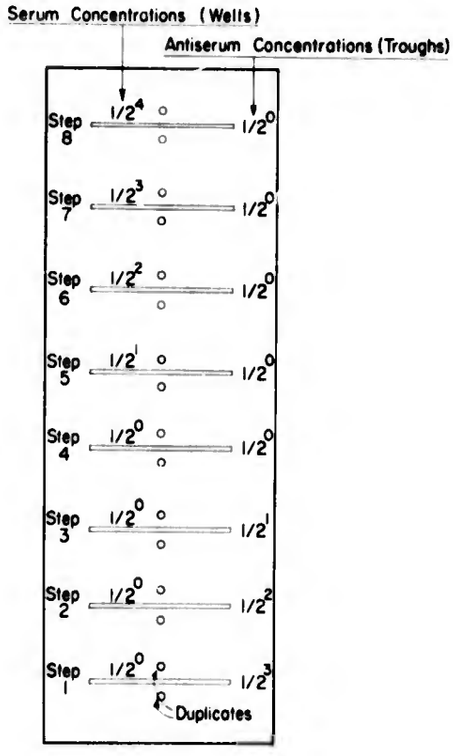

Figure 2. Location and concentration of serum and antiserum dilutions.

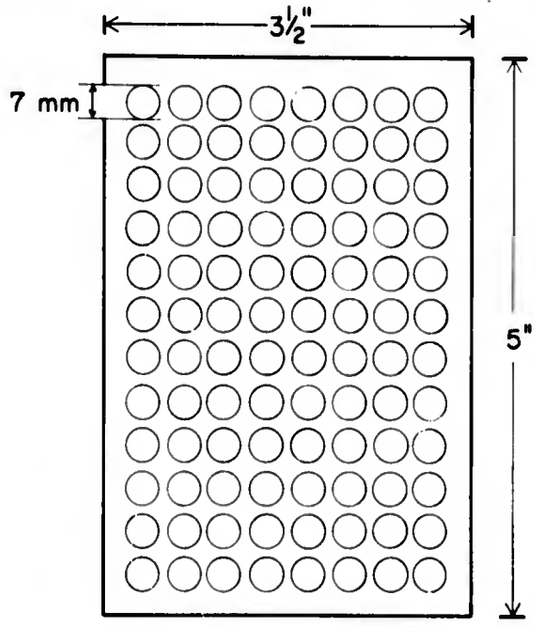

Figure 3. Configuration and dimensions of the serum dilution tray. Each hole is 1 cm deep.

"ladder of dilutions" until the sharpest point is found and recorded. This composite drawing in Figure 4 is also shown because the serum from no one individual will contain all the arcs in an adequate state for photographing.

Since the antiserum dilutions are always the same and more can be made up from the large stored pool, the location and end point for each protein can be established for normal serum. The antiserum then becomes a standard against which unknown serum can be compared with the normal pool. If the concentration of one protein should increase, its equivalence point will move up to a higher step in the "ladder of dilutions". Each protein moves independently. The important factor in the quantitation is a change in the position of the equivalence point from what is established for the normal pool, or if a previous value for a serum is known, it can be used as its own control for measuring subsequent changes. One plate of 8 steps with over 25 proteins delineated can usually be read and recorded after 24-48 hours and photographing, when desired, can provide a permanent record. Figure 5 shows a composite photograph of a complete 8SQ plate.

The antiserum contains many different antibodies for the different proteins and some are stronger than others. Those proteins which are antigenically stronger for the antibody producer will result in a higher antiserum titer. The stronger the antiserum is for a given protein, the lower the equivalence point will be on the "ladder". If the unknown protein is in very low concentration relative to the concentration of its antiserum or vice versa it may not precipitate at all. The dilution technique employed permits precipitation of protein which could not otherwise be visualized. Two advantages of this technique are delineation of more proteins and quantitative estimation. The reproducibility was tested by repeating the standard pool of serum interspersed randomly among unknown sera and it was found to be excellent.

The linearity of precipitation versus concentration was also excellent with this technique. This was evaluated by beginning with the highest concentration of albumin that gave a good equivalence point with the full strength antiserum and then serially diluting both the antigen and the antibody. The equivalence point was maintained throughout 10 two-fold dilutions at which point there were not enough reactants left to aggregate into a visible precipitate. This was repeated using the gamma G globulin arc and it was found to have the same linearity. The other proteins are assumed to be similarly linear but have not been specifically tested. The dilutions used are serial two-fold increments between the steps. This means if the end point shifts

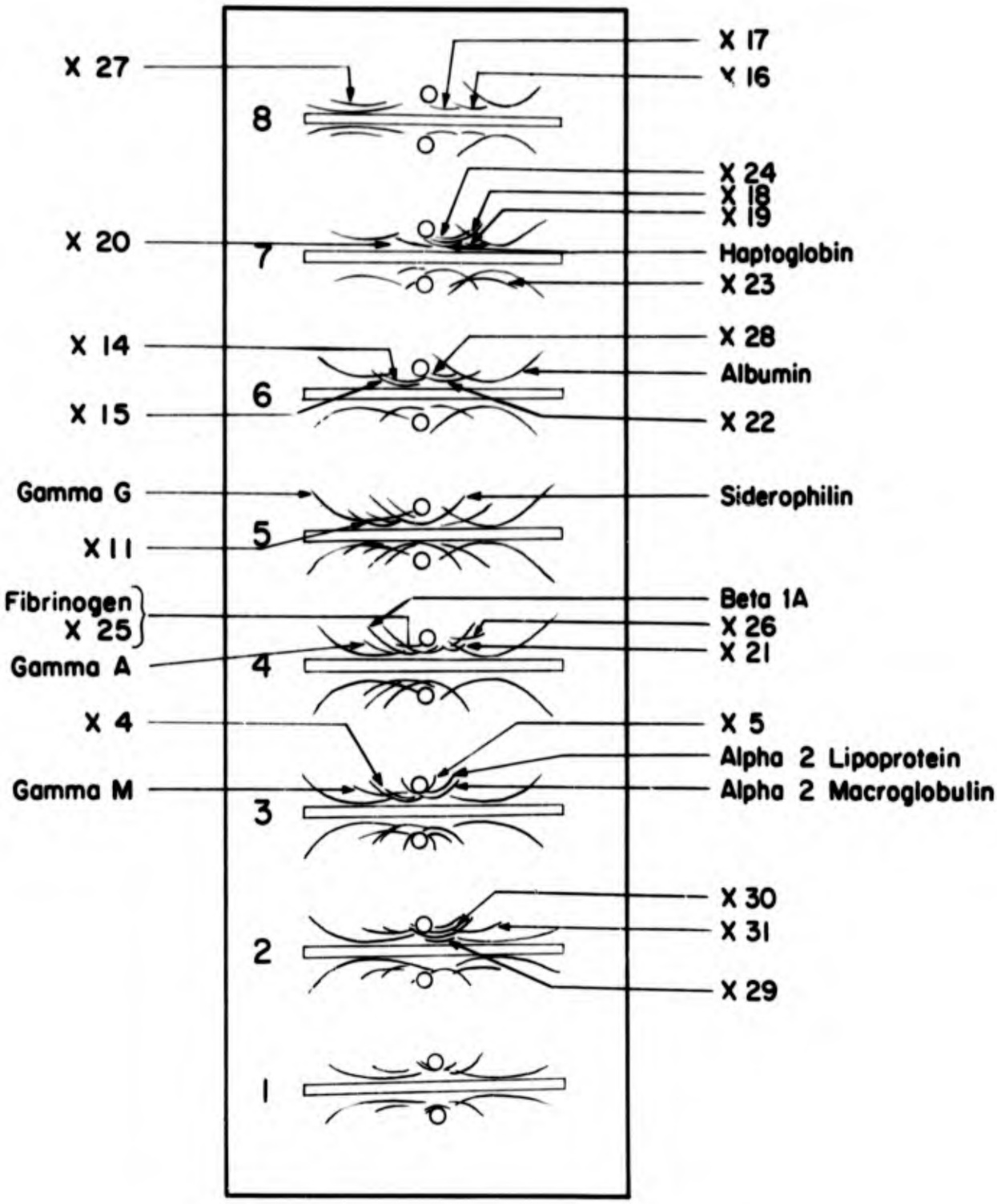

Figure 4. Artist's clarification of a complete 8SQ plate. The usual location of each protein end point is shown.

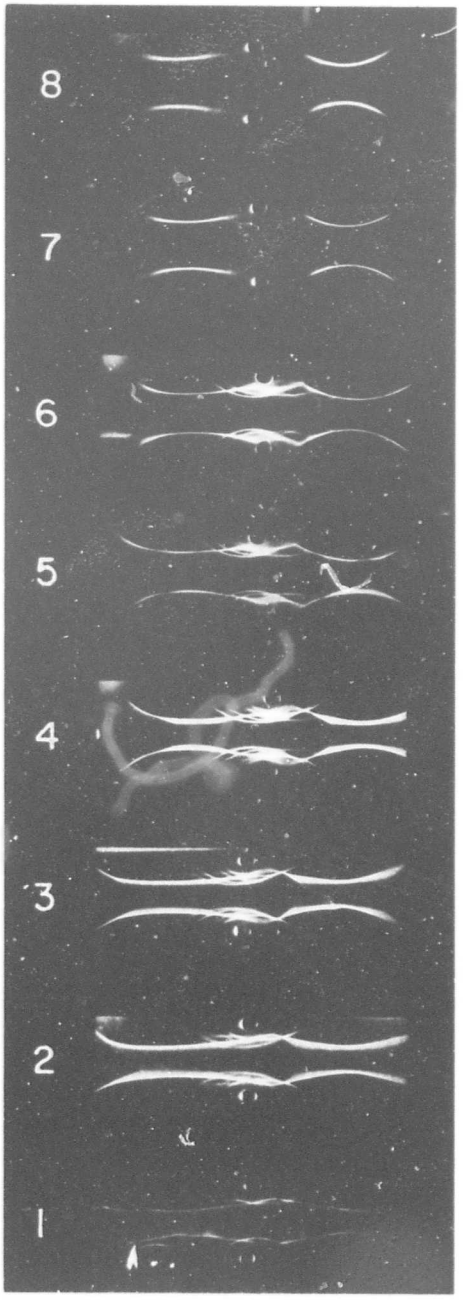

Figure 5. A composite photograph of a complete 8SQ plate.

up or down by one step, the concentration has doubled or halved respectively. This may seem to be a very large change for such a small difference in end points; however, it seems that most significant clinical changes in proteins are quite large. For example, Beta 1A globulin is normal when in step 4, borderline in step 3 and low below step 3. Often, however, it will not be seen at all when a patient has one of the complement lowering diseases.

When a small range and a greater accuracy is desired, the method is very versatile in that smaller increments of antibody dilutions can be prepared to detect smaller increments of change in the unknown. A broad screening method can be used in conjunction with a narrow range method with excellent efficiency.

As noted above, goat antiserum used in this laboratory gives quite satisfactory results; however, horse antiserum which has a very narrow precipitation range at the equivalence point would theoretically be the best reagent for use with this method.

The antiserum has been referred to as a secondary standard, that is, secondary to the antigen in the blood bank pool. It is being used here as a relative standard, but by running a purified protein of known concentration against whatever concentration of antiserum is selected for use, the equivalence point given by the antiserum effectively standardizes it. All of the antiserum dilutions can be assigned a calculated value and when the end point falls in a certain concentration that value can be assigned to the unknown. Therefore, the relative value method can be converted to an absolute value anytime a small amount of purified protein of known concentration can be obtained. The concentration need not exceed 100 micrograms per ml to give a good usable precipitin arc and only 5 microliters is required to fill one well. The lower limit of detectability is about 10 micrograms of protein per ml of serum. In addition, the work need not be delayed until the serum is standardized. Work can be done and relative values recorded. When that particular antiserum is standardized, all the previous values can be converted to absolute values.

Identification of the various protein arcs can be accomplished with either purified antigen or purified antibody. When purified antibody is used, it is placed as an extra well near the expected location of the unknown after electrophoresis. If these are identical, the unknown arc will join the known arc and no upward point will occur at the junction and there will be no crossing of the arc. If a purified antibody is used, the procedure is the same. In this situation the arc of the unknown

protein will turn sharply upward if it is identified by the purified antibody.

Two methods were devised to study the transport of beryllium in blood serum and to evaluate its ability to bind with serum protein. Immunoelectrophoretic analysis was done in both of these methods, followed in one case by Laser spectrographic analysis to detect non-isotopic beryllium, and in the other case, by autoradiography for radioactive beryllium 7(Be) using a modification of a previously reported method (ref.35).

The immunoelectrophoretic analysis was performed in the 8 slide quantitation configuration described. The slides were photographed and allowed to dry at room temperature, without washing, to avoid losing loosely bound beryllium.

Slides thus prepared from human and monkey sera, including positive and negative controls, were subjected to analysis for beryllium by Laser Microprobe spectroscopy. In these analyses the laser beam was discharged into various protein precipitate arcs wherever a 40 micron diameter area of specific precipitate could be viewed that included no other protein precipitates.

Radiographs were prepared from sera spiked with the radioactive beryllium and appropriate controls.

To prepare the radiographs, Kodak Royal Blue Medical x-ray film was cut to the size of the slides. This was done in a dark room, using only indirect lighting from a 7.5 watt bulb with a #2 Wrattan filter. Aluminum foil was cut large enough to wrap around a slide three times. The film was placed directly against the dried agar and wrapped around once with the aluminum foil. A piece of 1x3x8 inch polyurethane foam was placed on top of the wrapped slide and film to maintain uniform pressure of the film on the slide. The foam was squeezed tightly against the film and slide and the foil was wrapped twice more and folded, making sure that the ends were closed and no breaks had occurred in the wrapping.

The wrapped slides were sealed in black boxes with black photographic masking tape and kept in a sealed metal drawer during the exposure period.

B. Methods for Cellular Studies.

1. Sensitization of experimental subjects.

The study of cellular aspects of any immunologic response requires that the man or animal have the DHS type of hypersensitivity to the antigen to be studied. Although man is very easily sensitized to beryllium, animals such as guinea pigs and rabbits can be used for DHS studies although they are more difficult to sensitize and a number of experiments were necessary to accomplish useful levels of hypersensitivity.

a. Human.

No human subjects were deliberately sensitized for any of these studies. All lymphocyte donors were individuals who had been sensitized through natural exposures in their work and arrangements to obtain blood samples from volunteers were made through the private physicians in charge of the cases. Diagnosis of chronic beryllium disease had been made by clinical, laboratory, x-ray, and skin testing criteria.

b. Guinea Pigs.

Considerable time and effort was expended in attempts to sensitize guinea pigs to beryllium, chromium and tuberculin in order to obtain lymphocytes for the culture work. Our early attempts to sensitize the guinea pig to beryllium were no more successful than the reported efforts of Voisin (ref.14). Our approach was different from Voisin's to the extent that the beryllium solution was applied to the skin in a glycerol solution instead of subcutaneous or intramuscular injections with Freund's adjuvant. We hypothesized that Voisin had failed because he had not involved the skin in his sensitizing system, and that skin contained a factor or carrier necessary to the sensitization of the animal.

Unpublished work by Curtis (ref.36) indicated that beryllium fluoride would be the best compound to use for sensitizing the guinea pig. A solution of beryllium fluoride containing 2.13×10^{-1}M (1%) in glycerol was prepared and guinea pigs were treated on the skin of the abdomen. After three days of applications to the same area of each animal, moderate irritation developed. Continued application to the irritated area resulted in no further aggravation of the site. Subsequent application to other skin sites using the same concentration did not produce more rapidly developing irritation nor was any greater severity noted, and we concluded that this method did not result in sensitization. Due to the build-up of irritation in the surface application procedure, a better method of detecting sensitization was sought, and experiments were done to determine the non-irritating concentrations of an intradermal dose of 0.1 ml. A concentration of 1.5×10^{-4}M was negative in most animals and

only an occasional animal showed a 2-3 mm diameter inflammation. The 7.5×10^{-5}M concentration produced no irritation in any of the animals. Higher concentrations of 3.0×10^{-4}M and 6.0×10^{-4}M produced irritations of 5-10 mm in diameter that persisted for 5 days or more. Larger doses were included and some of these caused the area to turn white at first, and after several days a reddish border appeared and increased in diameter. Finally, the central white area became hard, similar to a third degree burn, and sloughed off. Several weeks wererequired for these areas to heal. The non-irritating concentration of beryllium sulfate was also determined in the samemanner and was found to be the same as BeF_2 on a molar basis. This was subsequently used for skin testing along with BeF in order to establish that hypersensitivity reactions were not in response to the fluoride ion.

A group of guinea pigs received intradermal injections of 0.1 ml of BeF_2 using the 6.0×10^{-4} M concentration to initiate sensitization. This dose was chosen because it was strongly irritating but produced a lesion which faded without necrosis. Injections were repeated at intervals of 3-5 days in rotating sites on the right side of the animals. In this manner at least one strongly irritated site was maintained for eight weeks and then the animals were skin tested intradermally with 0.1 ml of the 7.5×10^{-5} M concentration on the left side to avoid a retest reaction. A positive reaction, although less than 5 mm in diameter, was obtained in all animals. The animals appeared to have been sensitized but not strongly enough for use in lymphocyte culturing experiments.

Another attempt to adequately sensitize guinea pigs to beryllium was made using the method of Belman (ref.11,12).This consisted of preparing a 1% solution of BeF_2 in a mixture of methylcellosolve, water and Tween-80 (MCWT) in a proportion of 45:45:10, respectively. This solution was applied to guinea pigs on the clipped skin of the head and neck. The concentration was moderately irritating and it was applied once a week or more to maintain irritation in the area. These animals were skin tested intradermally at intervals and after eight weeks 10% of the animals gave a positive intradermal skin test to 1.5×10^{-4}M BeF_2 of 10x10 mm which was repeated in subsequent testing. Other positive skin tests of smaller size continued to be weak or decreased in subsequent testing.

The guinea pig maximization method (ref.37) was reported in a recent publication and was tried for beryllium. The method consisted of injecting some of the material with Freund's adjuvant, some without adjuvant, and applying some of the material to the surface in an absorbable form. All of these

procedures were done in the same area of the animal's back and an occlusive dressing was applied to the area. The surface application was renewed at intervals by injecting material through the dressing to keep an adequate concentration of sensitizer on the skin surface. The method required a two week interval to obtain sensitivity. At two weeks we found only weak intradermal sensitivity to beryllium again in 10% of the animals. The surface applications were continued after the occlusive dressings were removed, but only weak and questionable sensitivity could be found in the animals. The MCWT concentration of BeF_2 was increased to 10% without finding significant increase in the sensitivity reactions.

It can only be concluded at the present time that we have been able to obtain neither the degree of sensitization of guinea pigs reported by Belman (ref.11,12) and Polak (ref. 13) nor the 80% incidence that they have reported. Their strong positive reactions could possibly be due to retesting in the same area that they used to sensitize. Since they do not make this point clear, their results are open to some question. The retest reaction involves factors other than DHS, although some DHS factors may be involved.

Due to a poor response in our guinea pig sensitization experiments, we considered an attempt to increase the supply of sensitizable animals by inbreeding (ref.13) the few positive reactors that were obtained during the study. Unfortunately, there was only one strong positive female in our group so that inbreeding a strong reactor to a strong reactor would be possible in only one pair. Time did not permit further pursuit of this approach to the problem.

Although the sensitization of guinea pigs to beryllium has been disappointing, responses to chromium, tested at the same time, have been much more gratifying.

A potassium dichromate ($K_2Cr_2O_7$) solution of 6.8×10^{-3}M was prepared for the sensitization experiments. The guinea pigs were injected subcutaneously in the shoulder once a week for 2 weeks with 0.5 ml of the 6.8×10^{-3} M (0.2% chromium) solution that was homogenized with an equal volume of Freund's adjuvant. They were skin tested after one week using 1.02×10^{-3}M(0.03%)$K_2Cr_2O_7$ solution in saline and were already showing significant hypersensitivity. After 2 weeks 80% of the guinea pigs showed excellent reactions.

Guinea pigs were sensitized to tuberculin using Freund's adjuvant prepared with a heat killed H37Ra strain of mycobacteria. These injections were made subcutaneous anterior

to the scapula. The animals became sensitized after one week and reactions were even stronger after 2 weeks. The degree of delayed hypersensitivity must be evaluated before the cells can be used for other experimental purposes, and confidence in the existence of DHS in the animal is greatly increased by the ability to transfer this state to a non-sensitive animal with cells alone. Therefore, skin testing and cell transferring methods were used.

c. Rabbits.

Beryllium fluoride was applied to the clipped skin of the back of the rabbit's neck using a 1% solution in MCWT. In 3 weeks some moderate and a few strong positive intradermal skin tests had appeared in response to the 1.5×10^{-4}M Be concentrations. After 8 weeks, 80% of the animals had developed good DHS.

Patch tests were done with 3 concentrations of BeF_2 in MCWT to compare the intradermal tests. This was done because our experiences with the patch testing of animals did not conform with other reports (ref.11,12,13). These results further confirmed our previous experience. Even in the sensitized animals, the patch tests gave the same intensity of inflammation as they had in non-sensitized animals. The three concentrations of 2.0×10^{-4} M, 1.0×10^{-1} M, and 2.0×10^{-2}M did not give consistently distinguishable inflammatory reactions. In fact, often the lowest concentration produced more inflammation than the highest concentration. The intradermal skin tests gave more consistent results and did not give irritation reactions in control animals. Further evaluation of the epicutaneous method of skin testing is needed.

2. In Vivo Methods.

a. Skin Tests.

Skin testing by both surface application and intradermal injection was evaluated. Since retesting in a given area can give positive local reaction not reproducible in other areas of the skin, care was taken to maintain clear markings of all test areas. 1) Surface application:Test solutions of various concentrations were applied to areas 1/2 inch to 1 inch in diameter and an appropriate control solution was similarly applied nearby. The areas of application were delineated with ink or other marking liquid. No tape or covering patches were used for these tests. 2) Intradermal injections: These were done with 1 ml tuberculin syringes and number 25 gauge needles. Care was taken to get a well localized skin bleb characteristic of

intradermal injection, and all sites were marked with a sharp-pointed permanent liquid marker in the middle of each bleb as a locator.

b. Transfer Methods.

Animals used in transfer experiments were injected intraperitoneally with mineral oil and rested for 5 days to allow exudate cells to accumulate. The exudate was then removed by sacrificing the animal and emptying, then rinsing, the peritoneal exudate into a beaker. If the animal needed to be saved, the exudate was removed from an anesthetized animal using the tube from a peritoneal dialysis set designed for human use. This tube has multiple ridges and holes to avoid plugging, and balanced salt solution can be flushed in and out to remove the exudate without sacrificing the animal. Cells thus collected are washed with Hank's balanced salt solution and the cell concentration is estimated by counting or centrifuging techniques. Cell transfer is accomplished by intravenous, intraperitoneal or local skin injections into recipient animals.

3. In Vitro Methods.

a. Obtaining Cells.

Lymphocytes for culturing are obtained in several different ways, depending upon the species and site desired. For humans, peripheral blood is drawn using 1 ml of heparin (5,000 U.S.P. units/ml) for each 15 ml of blood. It is added to an equal volume of 2% gelatin or to a 10% volume of 6% dextran 250 and allowed to stand at 35.5°C. for 30-60 minutes. The leucocyte rich supernatant fluid is decanted with a Pasteur pipette into a sterile, capped, centrifuge tube. For guinea pig peripheral blood the animal is heparinized in advance with 1 ml of heparin. The animal is anesthetized with methoxyflurane inhalation anesthetic, the chest and abdomen clipped and sterilized with iodine and the blood removed by cardiac puncture. The animal dies of a combination of exsanguination and anesthetic overdose.The blood thus obtained is processed as the human blood. If spleen cells are being used, the exsanguinated animal is placed on its right side, draped with a sterile towel and a large incision is made in the abdomen and carried along a lower intercostal space. The exposed spleen is usually very well contracted and is easily removed. It is placed on an 80 mesh wire screen over a petri dish and the cells are teased out with forceps and flushed through the wire screen with minimum essential medium (MEM). The petri dish is filled with MEM to about 30 ml, the contents mixed well and allowed to stand one minute for any larger particles of debris to settle. The leucocyte rich

supernatant is decanted into a sterile, capped 50 ml centrifuge tube.

When guinea pig lymphocytes from lymph nodes are desired, the animal is prepared, including exsanguination as described above, and the cervical, axillary, prefemoral, inguinal or popliteal lymph nodes may be dissected out as desired and processed through the 80 mesh wire screen in a manner similar to the spleen, and the cell suspension is transferred to a sterile, capped 50 ml centrifuge tube.

The cells, from whatever source, are spun at room temperature at 1,000 rpm for 10 minutes in an International PR-2 centrifuge using the #269 head. The supernatant fluid is decanted and discarded and the cells are washed once with Eagle's Minimum Essential Medium. After washing, the cells are resuspended in asmall amount of the final medium.

b. Culture Methods.

Using a 0.1% crystal violet in 2% acetic acid as the counting fluid, total and differential cell counts were done simultaneously in the hemocytometer. The number of cells and the number of 0.1 ml cultures available with a concentration of 1.0×10^6 cells/ml were calculated. One ml aliquots were dispensed into sterile Falcon disposable polystyrene test tubes (#2003) using a disposable syringe and number 18 gauge needle. Plastic culture tubes and other plastic containers were heated at 60°C. in an oven for 3-5 days prior to use to eliminate traces of ethylene oxide which is extremely toxic to cultured lymphocytes.

The final medium used for cultured human cells consisted of 80 ml Eagle's MEM and 20 ml heated inactivated fetal calf serum with 1 ml L-glutamine (200mM) and 0.2 ml streptomycin (50 mg/ml). The same mixture was used for the guinea pig work with the addition of 0.4 ml of penicillin (100,000 units/ml). As advocated by some workers with animal cells (ref.38) the medium was enriched to approximately 40% with fetal calf serum but this did not solve our particular problem with animal cell stimulation. The various tubes of a culture set are treated with phytohemagglutinin or the antigen mixtures being studied such as PPD, beryllium, beryllium in extracts of skin or other proteins. The various additions to the cultures are used in volumes of 0.1 ml added to the cultures and the incubation carried out at 35-36 °C in a moist atmosphere of approximately 95% air and 5% CO_2 adjusted to maintain the desired orange color of the pH indicator in the medium.

Incubation time varied according to the experiments and the stimulant used. Cultures stimulated with PHA give optimal results when incubated for 3 days. Cultures stimulated with antigen seem to reach their optimum 5 days after incubation, although there may be different optimal times for different antigens.

The method above used fetal calf serum as part of the nutrient. This has been satisfactory for human cultures. Some authors have suggested human plasma gives better results, but since Tormey and Mueller (ref.39) have shown an inhibiting factor in the plasma of some humans, selection experiments are necessary. Pooled, aged plasma was tried, but was found to be toxic to the cells. The toxic factor was not established. A bottle of Rh positive plasma was obtained from blood stored 21 days and this has provided the best results in our experience so far.

c. Mitogens and Antigens.

Mitogens used in these experiments were phytohemagglutinin-M and phytohemagglutinin-P. Both are extracts from the red kidney bean, Phaseola vulgaris. These extracts are commercially available from Difco Laboratories. Phytohemagglutinin-M is adequate to stimulate human and some animal lymphocytes; however, phytohemagglutinin-P is a more purified preparation concentrating the active principle to about fifty times the concentration of phytohemagglutinin-M. This is able to stimulate some animal cells that fail to respond to phytohemagglutinin-M.

The antigen used in this work for establishing culturing technique and stimulating procedures was tuberculoprotein. The standard second test strength Purified Protein Derivative (PPD) was best for the purpose and was obtained from the Parke-Davis Company. Since both the buffered diluent and the PPD tablet contain a preservative, the tablet was dissolved in the same amount of MEM instead of the supplied diluent to avoid toxicity in the cultures. The amount of preservative in the tablet itself was not toxic to the cells. A solution thus prepared could be refrozen and used many times without significant loss of potency.

The metals used as antigenic components in the culturing system were prepared from BeF_2 , Cr_2SO_4 , $BeSO_4$ and $CrCl_3$. They were dissolved in Hank's balanced salt solution; the concentrations were limited to those that did not disturb the balance of the solution or the culture medium to which it was later added. The highest concentration that did not manifest

toxic effects on the cultured cells was taken as the initial concentration for evaluation.

Since the metal ions themselves were not expected to be antigenic, but rather some combination of the metal with a component of tissue, several methods were tried to combine or at least mix the factors.

Mixtures of tissues and skin slices were cut with scissors into pieces about 1 cubic millimeter in size and attempts were made to solubilize these using a Lordes homogenizer at 16,000 rpm. Both 15 ml and 50 ml size stainless steel homogenizing units were tried. Constant cooling was maintained by immersion in an ice water bath. The skin fragments in the mixture clumped around the blades and prevented the cutting effect of the homogenizer.

Although the Virtis 45 homogenizer gave better results, they were not entirely satisfactory. Electrophoresis of the supernatant liquid from such a homogenate showed characteristic staining for protein.

Grinding of these tissue fragments with sea sand in a mortar resulted in good disruption of the tissues. However, again, the crushed tissue formed into tough masses, this time with sand particles entrapped.

In the extraction attempts described, the supernatant fluid after centrifuging contained components that electrophoresed in agar and stained as protein.

Other ideas for obtaining or creating beryllium antigen that could not be tried here will be mentioned in the discussion section.

d. Harvest Methods.

The various tubes are harvested at appropriate times. The cells are first resuspended and a measured amount (15 drops) is transferred to a 10x75 mm test tube using a disposable pipette held at a45 degree angle for more uniform drop size. These cells are put aside for staining and morphologic studies. The remainder of the culture fluid is pulsed with 1.0 micro-curie /ml tritiated thymidine, uridine or leucine, depending upon the purpose of the suspension, mixed well and incubated at 35-36°C. for 2 hours. At that time, the radioactive sample is diluted 1:1000 with non-radioactive thymidine, uridine or leucine to compete with and essentially stop further uptake of isotope. Finally, the cells are spun down and the supernatant

radioactive culture fluid is decanted. At this point, the cells may be refrozen and stored until the full experiment is completed and then all may be processed together for liquid scintillation counting. If the pellet is not resuspended, the first washing step to be followed later coagulates the pellet which is then very difficult to solubilize.

Two basic methods may be used here. First, if total radioactivity is to be determined, the cells may be washed with cold 5% trichloracetic acid, then cold methanol, solubilized in hydroxide of hyamine and finally mixed into toluene scintillation fluid for counting. Second, if the specific activity is to be determined, the final solution of the material is aqueous rather than alcoholic and this requires the use of dioxane scintillation fluid (ref.40). The use of Cab-O-Sil greatly improves the efficiency of this two-phase system.

The toluene-base scintillation fluid was prepared by mixing 3 gm of 2-5 diphenyl-oxazole (PPO) and 100 mg of 1,4 bis[2-(5-phenyloxazole)] -benzene (POPOP) to one liter of toluene. Fifteen ml of this solution was drawn into a syringe and used to transfer quickly the digestion mixture into glass counting vials. The plastic test tubes became soft as a result of the contact with this solvent; however, no significant quenching of the subsequent counts resulted if any plastic dissolved into the counting vials.

The vials were counted in a Packard Tri-Carb liquid scintillation counter and the effects of quenching were evaluated by the channels ratio method of Herberg (ref.41). Experience showed that all cultures gave essentially the same amount of quenching so that the results usually could be expressed as direct counts per vial or as counts per culture tube.

e. Chemical Methods.

Since specific activity often gives results with less experimental variation, several experiments were done to evaluate this. Simultaneous chemical determination was done on an aliquot. The nucleic acids were measured by standard methods (ref.42). The DNA was prepared for extraction by first percipitating the sample with 2.0 ml of ice cold 0.5 N perchloric acid. This was allowed to stand for 10 minutes at 0 ° C. and then centrifuged. The precipitate was washed twice with 2.0 ml of ice cold 0.2 N perchloric acid. The final wash was discarded and the pellet extracted in 2.0 ml 0.5 N perchloric acid for 30 minutes in awater bath held at 70-75 ° C. The extracting volume may be varied depending on theDNA concentrations. At the end of 30 minutes the tubes were cooled in ice, spun and

the DNA concentration determined on the supernatant by the Burton Method. The diphenylamine reagent was prepared by dissolving 1.5 gm diphenylamine in 100 ml glacial acetic acid plus 1.5 ml concentrated sulfuric acid. This solution was stored in the dark. Immediately before use, 0.1 ml of a solution of acetaldehyde in water (16 mg/ml) was added to each 20 ml of diphenylamine reagent. To each 20 ml of DNA extract was added 4.0 ml of reagent and allowed to stand at 30° C. for 16-18 hours. An appropriate blank of 0.5 N perchloric acid was prepared with the unknown and all were read for color development on a Beckman DU spectrophotometer at 600 millimicrons.

The RNA was precipitated and washed in the same manner as for DNA. After the two washes with 0.2 N perchloric acid the pellet was dissolved in 2.0 ml of 0.3 KOH and incubated at 27° C. for 60 minutes. Then 1.25 ml of 1.2 N perchloric acid was added and allowed to stand in ice for 10 minutes before centrifuging. The RNA in the supernatant was determined by the orcinol method. A stock solution was prepared by dissolving 13.5 gm ferric ammonium sulfate and 20.0 gm orcinol in 500 ml H_2O. Immediately before use, 25.0 ml of the stock was added to 415 ml concentrated HCl and the mixture brought to 500 ml with distilled water. To 1.5 ml of the RNA extract, 4.5 ml of the reagent was added and heated for 20 minutes in boiling water. The density of color development was determined in a Beckman spectrophotometer set at a wave length of 600 millimicrons.

For measurement of the specific activity of cell protein, the cell protein was determined by the method of Lowry as adapted by Oyama and Eagle (ref.43) for use with tissue culture materials. The cell suspension was washed with balanced salt solution or saline, centrifuged and allowed to drain inverted over gauze or tissue. The cells were digested with the alkaline copper tartrate solution and an aliquot of the digest was then treated with the phenol reagent and allowed to stand for 30 minutes for color development. This was finally read in the Klett colorimeter using a 660 millimicron filter.

f. Method for Delayed Processing.

In some of the experiments that were planned there was an anticipated delay before processing of from four to five hours between the drawing of the blood and the time when it could be processed, since the patients lived over two hundred miles from the laboratory. The lymphocytes did not survive well in simple heparinized blood. After a number of experiments and some technical difficulties, we found that placing the heparinized blood into at least an equal volume of Eagle's MEM resulted in

good survival of the cells for at least five hours. Antibiotics added to MEM were especially important during this delay. Subsequently we established that even 24 hour delays could be well tolerated.

g. Evaluation of Delays After Processing.

Pogo (ref.44) claimed that a delay of 24 hours after processing before the addition of the mitogen resulted in a better response. We tried some experiments with this second delay by adding PHA to replicate cultures at daily intervals for 10 days. We did not get the improved stimulation that Pogo experienced, but obtained virtually identical values for the first 5 days, then experienced slight decreases by the tenth day. After the fifth day the control cultures began to show increased thymidine uptake even without stimulation. This phenomenon has also been reported by Sabesin (ref.45) but there has been little study of it. We have utilized the results of this experiment to require antigen and mitogen addition within 2-3 days and in actual practice it is almost always done the same day as processed.

h. Method for Guinea Pig Cells.

Guinea pig culture methods were essentially the same as those used in human cells. Various modifications are discussed in relation to results in Section III.

i. Method for Rabbit Cells.

The methods were essentially the same for rabbit cells as for human cells except that minimal essential medium containing Earle's balanced salt mixture rather than Eagle's seemed to give better results. The rabbit cells behaved similar to the human cells in this culture system and were much more satisfactorily maintained and stimulated than the guinea pig cells.

SECTION III

RESULTS AND DISCUSSION

A. Humoral Studies.

Sera from patients with chronic beryllium disease were studied to determine if they contained any of the humoral factors observed in some of the clinical conditions generally accepted as being auto-immune diseases.

Antitissue antibodies are seen in patients with systemic lupus erythematosis, rheumatoid arthritis and chronic active hepatitis and in some healthy relatives of such patients.

1. Beryllium Disease Factors Among Serum Proteins.

Sera from twenty human patients with chronic beryllium disease and sera from 40 monkeys were analyzed for the presence of various antitissue antibodies. The average normal value was obtained from a group of normal people. Of the first four specimens received, one contained a unique antinuclear membrane antibody when tested against thyroid tissue. Testing against skin and lung revealed the same results, but subsequent rechecks against five different thyroid tissues as well as further tests against skin and lung were negative. This patient had carcinoma of the breast in addition to chronic beryllium disease.

Several other specimens were weakly positive for antibodies against several tissue elements; however, no others contained antinuclear membrane antibodies. The weakly positive reactions were not regarded as significant. It is still possible that at some stage in the disease an antitissue antibody could have been present in high enough titer to be significant.

All of the monkey sera were negative for antitissue antibodies. The analysis of the rocket exhaust materials to which the monkeys were exposed has revealed it to consist of high-fired beryllium oxide which would be least likely to cause disease (ref.1). The negative results could also be explained by the possibility that if monkey antitissue antibodies were present, their cross-reaction with the human tissue substrate was not as good as the cross-reaction of our reagent antibody with the monkey immunoglobulins. Sera from eighteen patients and 40 monkeys were examined for the presence of antigamma globulin antibodies (Rheumatoid factors) using the latex fixation tests. One patient was strongly reactive. This was the same patient who had had carcinoma of the breast. Seven others were

definitely reactive. The sheep cell agglutination tests showed a significant titer (1:28) in only one of these, namely the patient who was strongly reactive for Rheumatoid factor.

Of the monkey sera specimens, only one was reactive and was weakly reactive. Sufficient quantity of these sera was not available to do a sheep cell agglutination test.

Some diseases associated with antigamma globulin antibodies are rheumatoid arthritis, pernicious anemia, silicosis with round nodular fibrosis, Hashimoto's thyroiditis and other connective tissue diseases. Since 44.4% of the cases tested gave a definite positive latex fixation reaction, this would suggest a connection between these conditions. However, two other factors must be considered. The sheep cell agglutination tests were essentially negative in the chronic beryllium disease patients, suggesting an antigamma globulin antibody without the true rheumatoid factor specificity seen in the other diseases, or else the titers were insufficient to be detected by the latter method. Age is known to be a factor in the latex fixation test in that elderly patients have a higher prevalence of positives. This factor was not relevant in this investigation since none of the positive subjects studied was over sixty-five years of age (where the high prevalence begins).

Thirty-five human sera and 50 monkey sera were screened by the IEPA-8SQ method for Beta 1A globulin, and all of these were within normal limits. Conditions with reduced levels of Beta 1A globulin are acute glomerulonephritis, lupus nephritis, and chronic active hepatitis. Even though it was not likely that the mechanism of chronic beryllium disease would be due to antigen-antibody reaction with binding of the third component of complement, this type of alteration when present is so clear cut and easily detected by the IEPA-8SQ method that it was worth checking. Both human cases and monkey subjects had normal levels of Beta 1A globulin, confirming the original impression that this factor would be normal. Gamma G, gamma M, gamma A and twenty-two other proteins were also screened by the IEPA-8SQ method.

Since gamma globulins are known to be elevated in chronic beryllium disease and gamma G is the major component, it might be expected to be elevated; however, only three cases showed a detectable elevation. These were all on steroid therapy which might have been responsible for the normal values. Gamma A globulin was slightly elevated in five cases, and gamma M elevated in five.

Among the monkeys, gamma G controls ranged from step 5.5 to 7.0 and none of the experimentals was clearly out of this range.

For monkey gamma A, most of the controls ranged from step 4.0 to 5.0 (one of the controls had no detectable gamma A). Four of the experimentals showed concentrations as low as step 3, but none was above the control range.

Although it is well known that in the active chronic disease there is elevation of the serum gamma globulin, the various different immunoglobulins now known to exist have never been simultaneously evaluated. One case with a very high gamma G also had the highest gamma A and gamma M in the group. So, all of the immunoglobulins can be affected by the pathological process. Most of these cases fell in the normal range, but since all these cases had been treated, and it is known that treatment lowers the immunoglobulin levels toward normal, there might be some detectable differences in new untreated cases. It would seem, however, that there is no clear cut tendency in the small number of cases studied here to suggest that measuring the individual immunoglobulin would be of more practical diagnostic value than measuring the total immunoglobulins. Since the factor measured in the latex fixation test is in the gamma M fraction, the observation of elevated levels in some of these sera is interesting, but there does not seem to be a direct correlation.

Finally, the screening of the other twenty-two serum proteins shows little deviation of these concentrations from that seen in normal people or pooled sera. Some proteins such as albumin, siderophilin, and alpha 2 macroglobulin are rather consistently present and havea narrow range of concentration. Other proteins, such as haptoglobin show a wider range and still others are inconsistently present. Some proteins which were consistently present in earlier samples became consistently absent later. This change was related to a change in the reagent antiserum pool and hemolysis in the specimens. Note that protein X25 which has been identified as fibrinogen is absent in these specimens since they are serum and not plasma. Proteins X26 and X27 are seen only in monkey serum. Trends cannot be recognized in the human or monkey specimens that would provide diagnostic or prognostic information.

It would seem that, other than the antigamma globulin antibody measured by the latex fixation reaction, these patients are almost totally lacking humoral changes relating them to autoimmune or connective tissue diseases. The results presented here, even though partly negative, do not change our view in that chronic beryllium disease is a continuing industrial

hygiene hazard and that pathogenesis is immunologic. Delayed hypersensitivity rather than humoral antibody mediation probably is the mechanism of pathogenesis.

2. Beryllium Transport by Serum Protein.

While working with serum factors, our interest was directed to the mode of transport of beryllium through the circulation. Beryllium has never been detected in the sera of patients with chronic beryllium disease (ref.46). The extreme sensitivity of the laser-spectrograph in conjunction with the ability of the immunoelectrophoresis to separate and concentrate serum proteins suggested a greater possibility of detecting minute amounts of beryllium if such were present.

Human and monkey sera were prepared by immunoelectrophoresis and then examined by a laser microprobe spectrometric analysis for the presence of beryllium in the serum. The immunoelectrophoreses separated the serum proteins into many individual arcs. Albumin, siderophilin, haptoglobin, Beta 1A, and gamma G globulin were separated well enough from other proteins to allow the laser beam to hit aportion of one arc without hitting any other arc. Known negative sera were used as negative controls and sera deliberately spiked with beryllium sulfate were used as positive controls. Preliminary results were encouraging in that most of the positive controls gave clear spectral lines for beryllium while all of the negative controls were negative. One of the human sera from a patient with beryllium disease gave a weak positive beryllium line when the laser beam was fired into the albumin arc. Subsequent attempts with the same serum have been negative and we now conclude that beryllium has still not been detected in the serum of patients. Its mode of transport from the lung to other parts of the body, if mediated by proteins in serum, is at a concentration that is undetectable. If transport is as a salt in serum, the concentration is below the limits of detection by the spectrographic method developed at the Kettering Laboratory (ref.46); perhaps the transport is effected by cells in the blood stream, namely the phagocytic cells.

Since most of the positive control sera exhibited good spectral lines for beryllium, it was interesting that some of the samples spiked with beryllium had somehow lost their beryllium before the laser analysis . In some of these, beryllium could not be detected in any of the precipitated protein arcs, nor could any be found in non-protein areas of the agar, even though it had been definitely added to the serum before analysis. In considering this finding, one possible explanation is that the electrophoresis of that group may have progressed longer

than the others, since this is not critical for the protein separation. This would have allowed the unbound beryllium to electrophorese completely out of the agar gel into the electrode wick and into the electrolyte bath, leaving undetectable concentrations in the gel. Because of this and a continued interest in the mode of transport of beryllium in the blood, a group of rat experiments was performed using intravenous radioactive beryllium ^{7}(Be) followed by immunoelectrophoretic analysis autoradiography.

Eighteen specimens were evaluated in the eight different antibody-antigen combinations of the IEPA-8SQ so that a total of 144 units were prepared. After the injection of 50 microcurie of ^{7}Be, blood was drawn from the animals at 1 hour, 17 hours, the protein arcs of the IEPA even after radiographic exposures of up to 16 months. In many of the radiographs, film was exposed along the right edge of the agar slide; however, this was artifac-tual due to excessive mechanical pressure exerted by the wrapping material. The failure to detect any binding of beryllium to serum proteins is significant from two points of view. First, the mode of beryllium transportation remains unclear, and second, the protein binding that is often necessary for antigen formation has not been demonstrated by these studies. Studies by others (ref.15) have suggested that beryllium may be transported through the circulation as the phosphate or the hydroxide. Particulates such as beryllium oxide may be transported by the phagocytic cells.

Belman, in studies of beryllium interaction with bovine serum albumin (ref.12) showed very little binding at a physiologic pH. He also showed good interaction of beryllium with ribonucleic acid (RNA) and desoxyribonucleic acid (DNA); thus, some transport can be effected by white blood cells when there is an abundance of these chemicals.

In situ binding with these chemicals in tissues exposed to beryllium, or beryllium binding where pH might be more favorable, may account for the beryllium antigen formation.

B. Cellular Studies.

1. Passive Transfer.

Several experiments were performed to attempt transfer of beryllium hypersensitivity to non-sensitive animals. In one of our attempts to transfer hypersensitivity to beryllium from a sensitized rabbit to a normal rabbit by means of the local transfer method (ref.47,48), we placed skin test antigen into the local transfer sites. We also placed skin tests into normal

skin sites about 1 inch away. At 24 hours the local sites injected with cell and antigen could not be distinguished from the control sites receiving cells and saline or cells and other antigen due mainly to the normal lymphocyte transfer (NLT) reaction. The skin test sites 1 inch away were negative as expected. We were very surprised, however, at 48 hours, when the skin test in normal skin became positive as the NLT reaction began to subside. This animal has been treated many times subsequently and is most exquisitely hypersensitive to beryllium both in the fluoride and sulfate forms, but it does not react to chromium. The appearance of these reactions at 48 hours is almost certain evidence of passive transfer of DHS and the persistence of the hypersensitivity on subsequent transfer experiments. This has been reported to occur by others (ref.47,48). In fact, it has also been used as a method to produce hypersensitivity since the cells give a good adjuvant effect. It seems from the reports that this works better if the adjuvant cells arefrom a sensitive animal rather than from a non-sensitive animal.

Another transfer experiment using a mixture of spleen cells and peritoneal exudate cells transferred intraperitoneally exhibited only a maximum skin reaction of 4x4 mm and this is less than usually desired for a positive response; however, investigators have based conclusions on 5x5 mm reactions, especially when the material is a weaker sensitizer in the first place.

Another passive transfer experiment was done and this time peripheral blood white cells and spleen cells from two rabbits giving 10x10 mm and 14x14 mm reactions respectively were pooled and injected intraperitoneally into a single recipient. A total of 4.37×10^8 lymphocytes were injected. An immediate skin test showed no reaction at 24 hours; however, the skin test proceeded to 3x3 mm and 5x5 mm in the next two days. Another skin test applied on the second day resulted in a 6x6 mm, then an 8x8 mm reaction which wasconsidered to be a moderately good positive reaction even though not as intense as desired. Two subsequent skin tests were weaker, as might be expected due to the transferred cells beginning to die. Since this was not a local passive transfer and the skin tests were not injected directly into the area containing transferred cells, active sensitization did not develop in these animals.

Rabbits were also easily sensitized to injections of chromium ($K_2Cr_2O_7$) in complete Freund's adjuvant using the same methods and skin testing as previoualy described for guinea pigs. Again, approximately 80% of the animals became clearly sensitized, but the others were rather weak.It is very interesting

that one animal was able to receive sensitizing doses over a period of several months without developing even the faintest DHS reaction to the chromium.

Several attempts to transrer the chromium sensitivity were made.To date, transfer of chromium hypersensitivity has not been accomplished with peritoneal exudate cells, lymph node cells, spleen cells or peripheral blood leucocytes. At first, cells from only one sensitized animal were used for transfer, then the cells from two animals were injected into one recipient. It is planned to pursue this further by collecting cells from 8-10 sensitized animals and transferring them into a single small animal. This transfer is very important in establishing delayed hypersensitivity (DHS) in an animal. If a transfer cannot be done in what is considered to be a clear cut and obvious DHS, then some new hypothesis will be needed to explain this difference from the classical bacterial hypersensitivity or DHS. We tested a group of guinea pigs sensitized to PPD to validate our methods. Transfer of this hypersensitivity with peritoneal exudate cells was accomplished without difficulty, indicating that the techniques that were being employed for the chromium work apparently were not defective, and that most likely, there is yet more to learn about the hypersensitivity state induced by the metal sensitizers.

2. An Attempt to Extract "Chromium Antigen".

Since chromium attaches to some tissue component, the reaction is believed to be immunologically specific for the complex thus formed (ref.49). Ifthis is the case, then if that complex might be obtained for use in the absence of free chromium, it should cause the same DHS reaction that the chromium itself causes in a recipient animal. To prepare such a complex the guinea pig was injected with 0.1 ml of 2.04×10^{-3} M $K_2Cr_2O_7$ in 10 separate intradermal sites. Four hours later it was believed that free chromium would have been largely carried away by the animal's own circulation and that only tissue bound chromium would remain. The skin sites were excised, pooled and part was extracted in saline by mincing and homogenizing. It was hoped that the extract thus prepared would give a positive skin test in sensitized animals. However, there was other irritating material in the extract and the control animals had reactions just as positive. This experiment is described briefly here because this is the type of approach that will eventually isolate the metal complexes or metal altered tissue components responsible for DHS reactions. These experiments should be continued. Further fractionation of this extract to remove the irritation factor may reveal the presence of the metal antigen complex.

3. Experience with Culturing of Lymphocytes.

Lymphocytes were found to grow rather easily in tissue culture after preliminary experiments revealed certain factors that were detrimental to their survival.

Many authors were reporting the use of polystyrene culture tubes while others were finding considerable difficulty with the same tube. Because of certain problems and inconsistent results, we switched to glass culture tubes that were disposable and prepared with an acid soak followed by distilled water rinsing. These were better, but there were still some inconsistent results that seemed mainly attributable to the culture tubes.

Another source of inconsistency was discovered to be dry heat sterilization of the glassware. Autoclave sterilization of the processing glassware with vacuum drying eliminated that problem, although the exact source of toxic factors was not identified.

Variation resulting from pH changes was controlled by use of a CO_2 controlled atmosphere incubator. Lowering the temperature from 37° to 35.5°C. was found to be of help, especially for animal cells.

With the controlling of these culturing factors, and perhaps a few others where the animals were involved, we were able to achieve a coefficient of variation of 15%±4% which is consistent with other workers with this technique at the present time.

There was only one major problem in harvesting of the cells. The method described for harvest included the final solution to the problem. However, since a large amount of time was expended working this out, and since others could have the same problem, it should be summarized in this report. The harvest step using hydroxide of hyamine involved enough water from the cultures so as to be insoluble in the toluene scintillation fluid. Accordingly, this was changed to the two-phase liquid scintillation counting method of Tye and Engel (ref.50) using Cab-O-Sil in dioxane, toluene and naphthalene. The Cab-O-Sil was used to absorb the aqueous phase and increase the efficiency. This worked so well that the cell digestion step could be done with 0.5 molar aqueous sodium hydroxide. While using this method with great success, suddenly a luminescence problem appeared. The samples counted millions of counts per minute and required days before significant differences could be detected between control and stimulated cultures. This was found to be related

to preparing the counting fluid mixture in advance. When this fluid was mixed with the Cab-O-Sil for more than a week, the luminescence could not be tolerated, whereas freshly prepared solution seemed not to be a problem. In the process of trying to find a better way to get the lymphocyte digest into the counting fluid, the method of eliminating the excess water with a methanol wash allowed the return to pure toluene scintillation fluid as described above. Where water cannot be eliminated the Tye and Engel (ref.50) method using freshly prepared solution is very effective, although somewhat more difficult technically.

a. Human Cells.

Much of the preliminary work referred to utilized human lymphocytes which are actually easier to work with than any of the animal cells. The results in individual experiments were recorded as absolute counts per minute; however, on different days, cells from the same donor were likely to give different absolute counts in both control and stimulated cultures. The ratio of stimulated and control cultures was more consistent, and therefore, results have been expressed as an experimental to control culture ratio (E/C ratio); thus, an E/C ratio of 1.0 represented a culture that did not stimulate at all.

The use of the E/C ratio as the unit of comparison permitted the consideration of labeling periods other than two hours since the control and experimental tubes should continue each approximately at its own rate for many hours. In an experiment to evaluate this, the E/C ratio was indeed essentially the same after two hours, five hours and 26 hours labeling periods. The time at which the labeling is started, however, is very critical and varies somewhat with the species. If the radioactive thymidine is added too soon, the cells are not yet metabolizing it fast enough for any significant uptake to occur before the thymidine is degraded into a non-metabolizable form. When this observation was first made, we thought that possibly the cells were beingdamaged by the radioactivity; however, we subsequently showed that the cells were quite capable of taking up tritiated thymidine at the proper time, even though the same amount of radioactive material had been added earlier and not taken up.

Stimulation of the human cultures with phytohemagglutinin-M (PHA-M) led to some interesting results in that different individuals gave widely different E/C ratios. For example, 6 individuals have E/C ratios of 1000,750,675,500,100 and 23. These ratios varied from time to time and seemed related to change in the serum portion of the culture media. Most often, a low ratio for a given individual was the result of having an

unusual elevation of the control value rather than a decrease in the value of the experimentally stimulated culture tube. This suggests that different batches of fetal calf serum may contain non-specific stimulating factors which have not been as yet identified. This is especially troublesome since the control value, being small, is much more seriously affected proportionally than the experimental values and a small change upward in the control value can markedly decrease the E/C ratio. Therefore, even the E/C ratio cannot be completely relied upon from one experiment to another. However, within a given experiment where all the factors are the same, the E/C ratio is the most reliable measure.

In some of our more recent work the E/C ratios are much lower than the ones obtained earlier and changing fetal calf serum has not improved the situation. In this regard, a bottle of AB Rh positive human plasma gives higher experimentals and lower controls than a parallel set-up with the same cells using the troublesome fetal calf serum. This may be of significant help in permitting comparison of different experiments in the future.

Stimulation of cultures by PPD gave much lower stimulation even in very sensitive cell donors than did PHA-M. For example, MH gave an E/C ratio of 2.7 and JR gave an E/C ratio of 6.1, LR gave 2.2 and BG showed 1.6.

Beryllium fluoride was used in the culture to determine its effect on cell metabolism as reflected by DNA synthesis. This was done with normal cells to establish beryllium doses that could be tolerated with lymphocytes. Since we would be dealing with the effect of beryllium on stimulated cells, a number of experiments were done where beryllium was added in varying concentrations to cultures that were also stimulated with PHA-M. In one such experiment, the beryllium fluoride concentrations used did not cause significant cell toxicity as shown in Table 1. This experiment suggests that the low beryllium concentrations may even be stimulating to normal cells. Beryllium is not expected to be a stimulant for normal cells in culture, but in this experiment, all the beryllium-containing cultures gave higher uptake of tritiated thymidine than the controls.

Looking at the beryllium effect from another point of view, separate control cultures were set up with each stimulated culture to contain the same beryllium concentration and hereafter this policy was followed. Table II, Experiment 1 shows counts per minute of tritiated thymidine uptake and E/C ratios where the non-stimulated control contained the same

TABLE I

Effect of beryllium on the uptake of tritiated thymidine on cultures stimulated by PHA-M.

Beryllium Concentration	Tritiated Thymidine Uptake CPM X 10^3
None	48.3 *
2.5×10^{-11}	65.3
2.5×10^{-10}	99.7
2.5×10^{-9}	56.2
2.5×10^{-8}	52.4
2.5×10^{-7}	64.7
2.5×10^{-6}	75.7
2.5×10^{-5}	92.6
2.5×10^{-4}	65.0

* Average of duplicates

concentration of BeF_2 as the stimulated culture. Again, beryllium was not toxic to the lymphocyte cultures. Note here that, again, there was greater uptake for all the beryllium-containing cultures. Not only did the cultures stimulated with PHA read higher values, but the non-beryllium containing cultures were also higher, resulting in little change in the ratios.

In another experiment lymphocytes were exposed in culture to higher concentrations of beryllium and the results shown in Table II, Experiment 2, indicate the graduation of beryllium effect from moderate to severe inhibition of the ability of the cells to divide. The ability to divide is clearly decreased at the 10^{-3}M Be concentration; however, this is only slightly reflected in the E/C ratio since both the control,and the PHA stimulated tubes are affected proportionally. The cultures containing the 10^{-2}Be concentration show not only severe reduction in the E/C ratio but also severe reduction in the actual uptake of the isotope - indicating severe toxicity. In other experiments the 10^{-3}M did not show toxic effects as severe as those reported above and it was regarded as the borderline toxic concentration.

TABLE II

Effect of beryllium concentration on thymidine uptake in PHA-M stimulated and non-stimulated cultures.

EXPERIMENT 1

Beryllium Concentration (Molar)	0	10^{-4}	10^{-3}	10^{-2}
CPM X 10^3 - PHA	141	152	189	
CPM X 10^3 - Control	3.82	4.30	4.92	
E/C	37	35.4	38.7	

EXPERIMENT 2

CPM X 10^3 - PHA	279	320	65.2	.254
CPM X 10^3 - Control	1.19	2.01	.513	.158
E/C	235	159	127	1.6

When beryllium is added to cultures of lymphocytes from patients with chronic beryllium disease, the

beryllium is considered an antigen and is added without other stimulants. Cultures of the same cells without beryllium are taken as the controls while other cultures are treated with PHA-M. Lymphocytes from patients with chronic beryllium disease were stimulated where sufficient cells were available, with PHA-M, Be at 10^{-3}M and 10^{-4}M and also with a skin extract in 10^{-3}M beryllium. Data from these cases are shown in Table III.

These data show that although the cells could be stimulated by PHA-M, no stimulation could be detected by the solutions containing the beryllium in the forms that had been prepared. In fact, in patient B the beryllium was severely toxic. At first appearance this would suggest an individual difference in human lymphocyte sensitivity to beryllium. However, the experiment that included patient B was performed on a different day from that of the others in Table III and control cells from a normal person showed similar toxicity. The source of this unexpected toxicity was not definitely identified, but fresh solutions were prepared for use in the human cultures and the toxicity was not observed in subsequent studies. Since the anticipated results with human beryllium disease did not occur, work with animals was initiated to clarify methods and to attempt to obtain responses to beryllium in cells from animals that were definitely hypersensitive.

b. Guinea Pigs.

It seemed likely in the work with human cells that the materials we were using as stimulants were not the antigens necessary to stimulate cells of patients with chronic beryllium disease or else that these patients' cells were not sufficiently sensitive to accept the stimulation. They could have decreased sensitivity with long duration of disease, or the steroid therapy in some could have decreased the lymphocyte responsiveness. Therefore, we elected to study more highly sensitive subjects, i.e. experimentally sensitized animals, to pursue this point further.

The most sensitive lymphocytes possible were needed to proceed with the search for a stimulating antigen. Since the literature (ref.36,37,43) indicated that guinea pigs could be easily sensitized by beryllium, and other reports (ref.48,51) indicated that guinea pig lymphocytes could be cultured, we proceeded first with setting up the lymphocyte culture technique for guinea pig cells. Only the latter two reports indicated success with guinea pig cells, so their methods were tried.

TABLE III

Effect of beryllium as an antigenic stimulant.

Patient		PHA-M	$Be10^{-4}M$	$Be10^{-3}M$ and skin	$Be10^{-3}M$
VM	E/C	6.6	-	0.94	0.91
MB	E/C	10.7	1.1	0.77	0.78
B	E/C	23.8	-	0.16	0.14

The cells were cultured easily; that is, they remained alive and were able totake up thymidine. However, when the PHA-M stimulant was added to the culture, depression rather than stimulation was noted. This same phenomenon occurred for guinea pig lymph node cells and spleen cells. Tritiated uridine was used to determine if RNA synthesis would provide any better indication of stimulation, but it did not. The toxic PHA-M was diluted to 50% and 25% strengths in the hope that a lower non-toxic concentration would provide stimulation. The lower concentrations allowed the cells to act more like normal cells, that is, less depressed, but stimulation did not occur.

Since we would be working with antigen in later experiments, we elected to try an antigen known to produce a response in a sensitized guinea pig. A guinea pig sensitized to mycobacteria H37Ra was sacrificed for spleen cells and stimulation was attempted with purified protein derivative. Control and stimulated cells were harvested on day 1 through day 6 and stimulation was not detected.

Different media were tried without improvement, replacement of media in the cultures every day or so afforded only minimal improvement, and an attempt to get a mixed lymphocyte stimulation (ref.52) failed. Trials with the newer PHA-P and Pokeweed mitogens showed them to be no better stimulants for guinea pig cells than PHA-M.

Guinea pig cells were not stimulated in any of the attempts. We have no explanation for this except to suggest that the PHA we were using was different in some way from that used by others who claim good stimulation. However, these experiments used several different lots of material and all gave similar toxic effects. Since these experiments were completed, there continue to be very few people reporting work

with guinea pig lymphocyte cultures, although the guinea pig is the most ideal animal for producing DHS to most antigens and would seem to be the most natural animal to use in this system. Since the termination of this contract we have achieved good stimulation of guinea pig lymphocytes with PHA-M and there has been no obvious change in our technique except for another change in the mitogen.

c. Rabbits.

Rabbit peripheral blood lymphocytes were easily cultured in the media described. Attempts to improve the system with human plasma rather than fetal calf serum failed. In fact, it was apparent that rabbit cells did not survive well in human plasma-supplemented media. Comparison of the two mitogens in an experiment showed that PHA-M gave an E/C ratio of 56.5 while the PHA-P was 14.9. Another experiment has been done using the beryllium solution prepared to stimulate the human cell cultures. The rabbit cells do not tolerate either the 10^{-3}M or the 10^{-4}M. The same procedure of establishing the maximum non-toxic dose would have to be done with the rabbit lymphocyte cultures as described for the human ones.

C. Suggestions for Further Study.

The humoral studies reported above have indicated the presence of an antigamma globulin antibody as shown by the high incidence of positive latex fixation reactions. The nature of the immuno-globulin should be pursued by collecting a quantity from patients with high levels and by purifying it for further characteristic studies.

The sera studied did not reveal any significant interaction or antibody factors with normal human tissues, yet the presence of elevated serum gamma globulin during the active chronic beryllium disease still suggests the presence of such factors. Such sera should be tested against tissue taken from patients with chronic beryllium disease. Biopsy or autopsy material, if preserved by freezing would be ideal for such study. If these immunoglobulins are not directed at normal tissue, they may be directed at beryllium-altered tissue. Also, lesions could be tested for the presence of immunoglobulins using the direct fluorescent antibody technique which would be another form of evidence that antibodies play a part in the disease process. In the cellular aspects of the study of chronic beryllium disease, we feel that considerable attention should be given to the claims of sensitization of animals where retest reaction could have been involved.

Much work is needed to better confirm the passive transfer of these DHS reactions by lymphocytic cells.

Other methods to obtain a useful, practical beryllium antigen might be considered. The method of using powdered tissue prepared by lyophilizing the tissue and then further freezing with liquid nitrogen and crushing to dry powder may provide a good method to deliver the beryllium antigen to the in vitro system. The use of whole tissue treated with beryllium or living tissue treated with beryllium in tissue culture may also provide methods to deliver beryllium antigen, known to be in the tissue, to the in vitro system.

The culture technology which has taken so long to apply to this situation should be pursued with these other ideas so that it may become the in vitro technique that is so seriously needed. The chemical nature of beryllium antigen in the usable, practical form suggested is still not understood, and further work needs to be done to characterize it.

Another important fact that should be evaluated for its very likely diagnostic value is that lymphocytes from sarcoidosis patients respond poorly in culture to PHA stimulation (ref.53). Since our studies have shown good response to PHA in the beryllium disease patients, this might become an important differential test in distinguishing these two clinically similar diseases.

We have expressed concern about the possibility of the patch test causing progression or activation of beryllium disease lesions (ref.54). It should be pointed out that Shima did an extensive industrial survey in Japan (ref.55) using a patch test and a study of his data showed that no one was sensitized by his patch test. There is no mention of activation of any potential or latent lesions. In tuberculosis, which is an analogous immunologic situation to chronic beryllium disease, we have no qualms about doing extensive human population surveys with the purified protein derivative of the tuberculosis organism. When done properly, this is a safe diagnostic tool, but when injected systemically, it can indeed cause the same type of activation of lesions that we fear for chronic beryllium disease testing. In light of these facts, perhaps some consideration should be given to finding a safer method of skin testing for chronic beryllium disease.

SECTION IV

CONCLUSIONS

A. Chronic beryllium disease patients can have a circulating antigamma globulin antibody as demonstrated by latex fixation test.

B. Patients can have an elevation of gamma A and gamma M as well asgamma G type of immunoglobulin during the active phase of the disease.

C. The marked elevation of the total immunoglobulin often seen in the active phase of the disease would, theoretically, be directed at some antigen present or formed somewhere in the body. However, other than that noted above, this study has not identified such an antigen in normal tissues.

D. Guinea pigs have been sensitized using epicutaneous doses of beryllium in MCWT. In these studies sensitization has been demonstrated by intradermal challenge in another area of skin thus avoiding questions and doubts about retest reaction.

E. Rabbits can be sensitized by using the same methods as for guinea pigs. Although the rabbit is not usually considered ideal for research of this type, it showed a greater intensity of hypersensitivity than produced in the guinea pig and a greater percentage has been sensitizable.

F. Passive transfer of hypersensitivity by means of cells, important in evaluation of DHS phenomenon, has been demonstrated by the local transfer method in one rabbit and, less convincingly, by the systemic transfer method in another rabbit.

This lends considerable support to the concept that beryllium hypersensitivity is a DHS phenomenon, but this should be repeated with larger numbers of animals.

G. In vitro methods have been adapted for maintainance and stimulation of human, guinea pig, and rabbit lymphocytes in tissue culture. These lymphocyte culture techniques are directly suited for use as in vitro methods for detecting DHS in man and animals where it is believed that the use of skin testing procedures may be dangerous.

H. Our work has shown that lymphocytes from patients with chronic beryllium disease can be easily stimulated by PHA. There are other reports (ref.53) showing that lymphocytes from patients with sarcoidosis are markedly unresponsive to PHA stimulants.

PHA responsiveness of lymphocytes would be a most important criterion in the differential diagnosis of these two very similar diseases.

I. These studies do not propose to change any of the recommendations made in earlier industrial hygiene reports (ref.56,57) published by this laboratory.

REFERENCES

1. Spencer,Howard C.et al.,"Toxicological Studies on Beryllium Oxide and Beryllium Containing Exhaust Products",Aerospace Medical ResearchLaboratory, Technical Report No.68-148,1968.

2. Cholak, J., L. Schafer,D.Yeager, "Exposure to Beryllium in a Beryllium Alloying Plant", Am.Ind.Hyg.Assoc.J,28:1967.

3. Tepper, L.B.,H.L.Hardy, and R.I. Chamberlain, Toxicity of Beryllium Compounds, Elsevier Publishing Company, page 2,1961.

4. Hardy, H.L. and I.R. Tabershaw, "Delayed Chemical Pneumonitis Occurring in Workers Exposed to Beryllium Compounds". J. Ind.Hyg. 28:199,1946.

5. DeNardi,J.M., Chronic Pulmonary Interstitial Granulomatosis. AMA Arch.of Ind.Hyg, and Occup.Med. 3:543,June,1951.

6. Sterner, J.H., and Eisenbud, M., Epidemiology of Beryllium Intoxication.AMA Arch. of Ind. Hyg. 4:123-151,August,1951.

7. Curtis, G.H.,:Cutaneous Hypersensitivity Due to Beryllium; A Study of 13 Cases. AMA Arch. of Derm. and Syph. 64:470-482, October,1951.

8. Van Ordstrand, H.S., The Twilight Zone of Allergy. Annals of Allergy, Vol.23,Pages 262-264, June,1965.

9. Hardy,H.L.,: Beryllium Poisoning - Lessons in Control of Man -Made Disease. New Eng. J. of Med.273:1158-1199,November,1965.

10. Miller, J.F.A.P., G.F. Mitchell, "Interaction Between Thymus or Thoracic Duct Lymphocytes and Bone Marrow Derived Cells in an Immunologic Response". Federation Proceedings 27:265,1968.

11. Belman, S.,Beryllium Binding of Bovine Serum Albumin and Epidermal Constituents. Dissertation for Doctor of Philosophy at New York University, 1958.

12. Belman, S.,"Beryllium Binding of Epidermal Constituents". J. Occup. Med.4:175,1969.

13. Polak, L.,J.M. Barnes and J.L. Turk, "The Genetic Control of Contact Sensitization to Inorganic Metal Compounds in Guinea Pigs". Immun. 14:707,1968.

14. Voisin, G.A., A. Collett, H. Daniel-Moussard and Toullet,F., Rev. Franc d'etudes Clin. et Biol. 9:819,1964.

15. Reeves, A.L., Vorwald, A.J.,"The Humoral Transport of Beryllium". J. Occup.Med. 3:567,1961.

16. Tepper, L.B.,H.L. Hardy and R.I. Chamberlain, Toxicity of Beryllium Compounds. Elsevier Publishing Company,page 53, 1961.

17. Friou,G.F., S.C. Finch, and Detre,K.D., Interaction of Nucleic and Globulin from Lupus Erythematosis Serum Demonstrated with Fluorescent Antibody, J. of Immun. 80:324-329, April,1958.

18. Chadwick,C.S.,and J.E. Fothergill,"Fluorochromes and Their Conjugation with Proteins", in Narin,R.C.,Fluorescent Protein Tracing, Chapter 2, Pages 4-30, Edinburg and London, Great Britain; E&S Livingstone,Limited,1962.

19. Ziff,M., The Agglutination Reaction in Rheumatoid Arthritis. J. of Chronic Diseases. 5:644-667,June,1957.

20. Schroeder,W., E.C. Franklin, C.M. McEwen,"Rheumatoid Factors in Patients with Silicosis with Round Nodular Fibrosis of the Lung in the Absence of Rheumatoid Arthritis", Arthritis and Rheumatology 5:10,1962.

21. Roitt, I.M. and D. Roniac, Thyroid Autoimmunity, British Medical Bulletin, 16:152-158 (May-August),1960.

22. West, C.D.,J.D. Northway, and N.C. Davis, Serum Levels of Beta 1 Globulin, a Complement Component in the Nephritides,Lipid Nephrosis and Other Conditions. J. of Clinic. Inv. 43:1507-1517, August,1964.

23. Alekseyeva,O.G., A.P. Valova,N.V. Svinkina, "The Mechanism of the Effect of Beryllium on the Organism", Farmakologiya i Toksikologiya 3:353,1966.

24. Freiman, D.G., "Pathologic Change of Beryllium Disease". AMA Arch.Ind. Health.19:No.2,1pages 188-189,1959.

25. Alekseeva, O.G., "Ability of Beryllium Compounds to Cause Allergy of the Delayed Type', Gigiene Touda Professional'nye Zabolevaniya, Volume 9, No. 11, Page 20, 1965.

26. Arnason, B.G., and B.H. Waksman, "The Retest Reaction in Delayed Sensitivity". Lab. Invest. 12:737,1963.

27. Dolby, A.E.,J.J. Williamson, and D. Adams, The Retest Contact Hypersensitivity Reaction in Guinea Pig Skin and Oral Mucosa. Br. J. Exp. Path. 50:343,1969.

28. Hill, W.C., "The Influence of the Cellular Infiltrate on the Evaluation and Intensity of Delayed Hypersensitivity Reactions". J. E. M. 129:363,1969.

29. Chiappino , G.E., C. Vigiliani, "Delayed Type of Hypersensitivity Reactions to Beryllium Compounds. Arch. Path. Vol.87:131-140,1969.

30. Van Ordstrand, H., J.F.Zielinski, J.M. DeNardi,"Medical Management of Beryllium Workers" in H.E. Stokinger's Beryllium, Its Industrial Hygiene Aspects, Edition I, New York Academic Press, Chapter 10, Pages 323-327, 1967.

31. Scheidegger, J.J., Une Micro-Methode de l'immuno-electrophorese. Int. Arch. of All.. 7:103-110,August,1955.

32. Ziff,M.,et al.Agglutination and Inhibition by Serum Globulin in the Sensitized Sheep Cell Agglutination Reactions in Rheumatoid Arthritis. Am.J.Med. 20:500-509, April,1965.

33. Nowell, P.C., Phytohemagglutinin - An Initiator of Mitosis in Cultures of Normal Human Leukocytes. Cancer Research 20:462.1960.

34. Pearmain, G., R.R. Lycette, and P.H. Fitzgerald, Tuberculin Induced Mitosis in Peripheral Blood L eucocytes. Lancet I. 637,1963.

35. Jett,R., J.O. Pierce,K.L. Stemmer, "Toxicity of Alloys of Ferrochromium". Arch. Environ. Health . 17:29-34,1968.

36. Tepper, L.B.,H.L. Hardy, and R.I. Chamberlain, Toxicity of Beryllium Compounds,Elsevier Publishing Company, page 104,1961.

37. Magnusson, B., A.M. Kligman, "The Identification of Contact Allergens for Animal Assay, The Guinea Pig Maximization

Test", J. Invest.Dermat. 52:268-276,1969.

38. Oppenheim, J.J., R.A. Wolstencraft, and P.G.H. Gill, Delayed Hypersensitivity in Guinea Pig to a Protein Hapten Conjugate and Its Relationship to In Vitro Transformation of Lymphnode, Spleen, Thymus and Peripheral Blood Lymphocytes. J. Immun. 12: 89,1967.

39. Tormey, D.C. and G.C. Mueller, "An Assay for the Mitogenic Activity of Phytohemagglutinin Preparations". Blood. 26,Number 5.

40. Tye,R., J.P. Engel, Liquid Scintillation Counting of Carbon -14 in Agueous Digests of Whole Tissue. Anal. Chem. 37:1225,1965.

41. Herberg,R.J.,"Channels Ratio Method of Quench Correction in Liquid Scintillation Counting". Packard Technical Bulletin, No.15,1965.

42. Methods of Biochemical Analysis, Volume 14, The Determination of Nucleic Acids, Page 113, Interscience Publishers, A Division of John Wiley and Sons, New York, London and Sydney, 1966.

43. Oyama, Vance I., H. Eagle. Measurement of Cell Growth in Tissue Culture with a Phenol Reagent. (Folin-Ciocalteau). Proc.Soc.Exp.Biol.Med. 91;305,1950.

44. Pogo, B.G.I., V.G. Allfrey and H.E. Mirsky, RNA Synthesis and Histone Acetylene During the Course of Gene Activation in Lymphocytes. Proc.Nat.Acad.Sci.55:805,1966.

45. Sabesin, S.M., "Lymphocytes of Small Mammals: Spontaneous Transformation in Cultures to Blastoids". Science. 149: 1385,1965.

46. Cholak, J.,"The Analysis of Traces of Beryllium", AMA Arch. Ind.Health,19,No.2, Pages 205-210,1959.

47. Metaxas, M.N., and Buehler Metaxas, "Studies on the Cellular Transfer of Tuberculin Sensitivity in the Guinea Pig". J. Immun.75:333,1955.

48. Chase, M.W., "The Cellular Transfer of Cutaneous Hypersensitivity to Tuberculin". Proc.Soc.Exp.Biol.Med.59:134,1945.

49. Samity, M.H.,and S. Katz,"A Study of the Chemical Reactions Between Chromium and Skin", J.Invest.Dermat. 43:35,1964.

50. Tye,R., and J.D. Engel,"Liquid Scintillation Counting of Carbon in Aqueous Digests of Whole Tissues", Anal.Chem. 37:1225,1965.

51. Sabesin, Seymour, Blastoid Transformation of Rabbit and Guinea Pig Peripheral Lymphocytes in Phytohemagglutinin. Proc.Soc.Exp.Biol.Med.122:351,1966.

52. Bain, B., M.R. Vas, and L. Lowenstein,"The Development of Large Immature Mononuclear Cells in Mixed Lymphocyte Cultures", Blood. 23:108,1964.

53. Chase, M.W., Delayed Type Hypersensitivity and the Immunology of Hodgkin's Disease, with a Parallel Examination of Sarcoidosis. Cancer Research 26:1097,1966.

54. Sneddon, I.B., Berylliosis, A Case Report.Medical Journal 1:1448,1955.

55. Shima, S., "Beryllium Hazards and the Sensitization Phenomenon Due To Beryllium Exposures, with Special Reference to the Patch Test". Dept. of Public Health, Nagoya City University Medical School, Nagoya, Japan, 1966.

56. Dept. of Environmental Health, Kettering Laboratory, Cincinnati, Ohio, Technical Report No. ASD-TR-62-665, April,1962,Contract AF-33600-37211.

57. Dept. of Environmental Health, College of Medicine, Cincinnati, Ohio, Critique of Current Safety Practices, No. AMRL-TDR-64-75, September 1969.