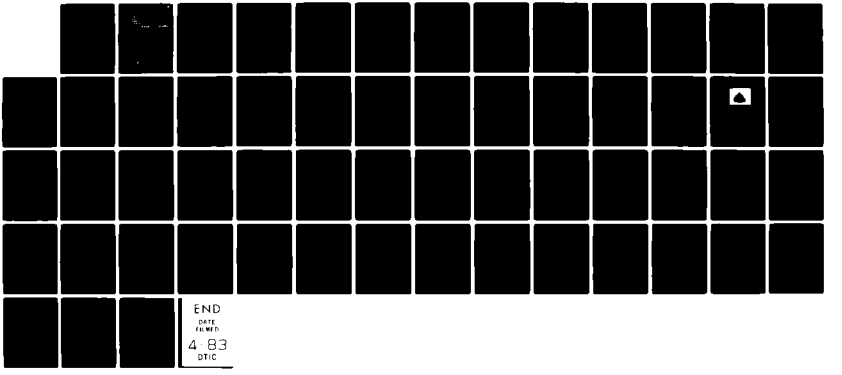
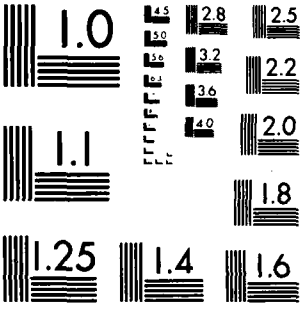
AD-A126 410 MOLECULAR INTERACTIONS OF HIGH ENERGY FUELS AND JET FUELS WITH ONCOGENIC..(U) OHIO STATE UNIV RESEARCH FOUNDATION COLUMBUS J R BLAKESLEE FEB 83 1/1

UNCLASSIFIED AFOSR-TR-83-0139 F49620-80-C-0087 F/G 6/2 NL



END
DATE FILMED
4-83
DTIC



MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS-1963-A

AFOSR-TR- 83-0139

AD A126410

RF Project 762179/712882
Progress Report

the ohio state university
research foundation
1314 kinnear road
columbus, ohio
43212

MOLECULAR INTERACTIONS OF HIGH ENERGY FUELS AND JET FUELS WITH ONCOGENIC VIRUSES AND ENDOGENOUS VIRUSES

James R. Blakeslee
Department of Veterinary Pathobiology

For the Period
July 31, 1981 - December 31, 1982

AIR FORCE OFFICE OF SCIENTIFIC RESEARCH
Directorate of Life Sciences
Washington, D.C. 20332

Contract No. F49620-80-C-0087

DTIC FILE COPY

DTIC
ELECTE
APR 6 1983
D

February, 1983

Approved for public release;
distribution unlimited.

FEB 18 1983

83 04 0

Unclassified

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER AFOSR-TR- 83-0139	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) MOLECULAR INTERACTIONS OF HIGH ENERGY FUELS AND JET FUELS WITH ONCOGENIC VIRUSES AND ENDOGENOUS VIRUSES		5. TYPE OF REPORT & PERIOD COVERED Progress 7/31/81-12/31/82 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) James R. Blakeslee		8. CONTRACT OR GRANT NUMBER(s) F49620-80-C-0087
9. PERFORMING ORGANIZATION NAME AND ADDRESS The Ohio State University Research Foundation, 1314 Kinnear Road Columbus, Ohio 43212		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 61102F 2312/A5
11. CONTROLLING OFFICE NAME AND ADDRESS AIR FORCE OFFICE OF SCIENTIFIC RESEARCH/NL Building 410 Bolling Air Force Base, D.C. 20332		12. REPORT DATE February, 1983 13. NUMBER OF PAGES 54
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report) Unclassified 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

In vitro Assays, Carcinogens, ST Feline Sarcoma Virus, Virus Transformation, Hydrazines, Jet Fuels, Polycyclic Hydrocarbons, Aromatic Amines, Mycotoxins, Aminofluorenes, Provirus, RD114 Virus, RD114 P28

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The objectives of this research are to develop rapid in-vitro assays to evaluate the carcinogenic potential of chemicals used by the U.S. Air Force. Snyder-Theilen Feline Sarcoma Virus (ST FeSV), quantitatively transforms human skin fibroblasts following second order kinetics. These studies were performed in order to determine whether chemicals altered ST FeSV transformation in a predictable manner and to correlate the alteration with the carcinogenic or non-carcinogenic activity of the text chemical.

(continued)

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

Unclassified

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

Block 20--Abstract (continued)

The results, to date, show diverse carcinogens classed as: aromatic amines, polycyclic hydrocarbons, Aminofluorenes, hydrazines, asbestos and mycotoxins inhibited virus transformation when virus infected cells (2 hours post-infection) were exposed to test chemical, while non-carcinogenic chemicals had no significant effect on transformation. Triton X-100, acetone, petroleum and shale oil derived JP5; RJ5 and diesel fuel, marine, demonstrated non-carcinogenic activity while formaline demonstrated carcinogenic activity. Experiments designed to show the specificity of the antagonistic effect of known carcinogens are reported. Disulfuram inhibits biotransformation of 1,2 symmetrical dimethyl hydrazine (SDMH) metabolites, azomethane to azoxymethane (ultimate carcinogen) thereby preventing carcinogenic effect of the proximate carcinogen SDMH.

Cells treated with SDMH inhibited virus transformation whereas cells co-treated with SDMH and disulfuram resulted in no significant difference in transformation frequency when compared to controls. Furthermore, the data show disulfuram failed to abrogate the inhibitory effect of MAMA, the ultimate carcinogen of SDMH.

Detailed methodology required to ascertain effect of chemicals on ST FeSV provirus integration and synthesis are presented. These studies will eventually elucidate mechanisms of chemical-virus interactions.

A second in vitro assay is described. Isolation and purification of an endogenous feline virus, RD114 structural proteins designated P28, is described. P28 is expressed in all feline tissues and cells. Data show increased P28 expression resulted following in vitro exposure to feline embryo cells with a napthylamine and a polyaromatic hydrocarbon. The levels of expression were significantly greater than control values.

Progress Report

to

The Air Force Office of Scientific Research
Directorate of Life Sciences
Bolling Air Force Base
Washington, DC 20332

Title: Molecular Interactions of High Energy Fuels and Jet Fuels

with Oncogenic Viruses and Endogenous Viruses

Inclusive Dates of Report
July 31, 1981 to December 31, 1982

Submitted by:

James R. Blakeslee, Jr., Ph.D.
Associate Professor
Department of Veterinary Pathobiology
The Ohio State University
1925 Coffey Road
Columbus, Ohio 43210
Telephone (614) 422-5661

Accession For	
NTIS GRA&I	☒
DTIC TAB	☐
Unannounced	☐
Justification	
By	
Distribution/	
Availability Codes	
Dist	Avail and/or Special
A	

DTIC COPY INSPECTED

The views and conclusions contained in this document are those of the authors and should not be interpreted as necessarily representing the official policies or endorsements, either expressed or implied, of the AFOSR of the U.S. Government.

I. Research Objectives

A. The objectives of this research are to:

1) Develop rapid in vitro assays to evaluate toxicologic and carcinogenic potential of chemicals used in world-wide U.S. Air Force operation. Rapid short-term assays to evaluate and determine toxic effects and carcinogenic potential would greatly reduce the time and money now required to determine these same effects in in vivo animal systems.

This research has involved the development of two systems, both involving in vitro assays. One system has been used to investigate the effect diverse chemicals have on a feline sarcoma virus transformation of human cells. The results of these experiments show chemical carcinogens specifically inhibit FeSV transformation when virus-infected cells are exposed to chemicals between 2 and 6 hours post-infection, while non-carcinogenic chemicals do not.

The second system has been used to investigate and correlate carcinogenic potential with gene expression of an endogenous feline virus (RD-114). To date, carcinogens increase expression (ng virus protein/mg cellular protein) in a time and dose-dependent manner.

The first system is being validated as to specificity and sensitivity by a double blind study in conjunction with the Chemical Research Resources Program, CPCB, DCCP, NCI.

The validation of such assays may establish predictable parameters of potential carcinogens and lead to the understanding of complex interactions between normal cellular processes and environmental carcinogens.

B. Specific Objectives

The specific aims of this contract period are to:

1. Expand and validate ST-FeSV transforming assay system with known chemicals and 24 unknown computer coded chemicals in a double-blind study coordinated with NCI, NIH.

2. Expand classes of chemicals to include carcinogens and non-carcinogens in determining whether a predictive assay will result from the studies in which expression of the endogenous feline virus, RD-114, translational protein P28 are evaluated following chemical treatment of feline cells.

3. Determine effects of chemical treatment on proviral integration and synthesis through radiolabeled probes. These studies will elucidate molecular mechanism(s) of chemical-virus interactions.

II. Status of Research

A. Cell lines and viruses.

The primary cell line used for the oncogenic virus Snyder-Theilen Feline Sarcoma Virus Transforming Assay System, was $Detroit_{550}$ human skin fibroblast cells. These cells from American-Type Culture Collection, and designated CCL 109 are used between passage levels 16 and 30. They have been characterized and stored in liquid nitrogen in order that these passage levels can be used. The cell line RD-114/RD, a human rhabdomyosarcoma cell line shedding the RD-114 virus, is used for the isolation and purification of the P28 glycoprotein. Pools of Snyder-Theilen strain of feline sarcoma (leukemia) virus were grown and titrated in $Detroit_{550}$ cell lines. These cell lines have been routinely screened for the presence of mycoplasma and bacteria and other viruses.

B. Endogenous virus (RD-114) studies.

The radioimmunoassay for determination of P28 expression has been standardized and several chemicals have been evaluated for dose and time effects (see Results of experimentation).

C. Molecular virology studies.

Conditions for growing FeLV have been established and co-carcinogenesis studies were completed with 6 chemicals, and most major problems have been resolved.

D. Results of experimentation.

1. Effect of chemicals on RD-114 P28 expression.

The RD-114 virus is an endogenous xenotropic feline virus which is partially expressed in all normal cat cells. A structural protein, P28, a translational gene product of the RD-114 virus can be detected in all normal feline tissue. In this study we intend to determine if a significant increase or decrease occurs following treatment of feline cells with a variety of carcinogenic and non-carcinogenic compounds.

P28 levels are determined using a competitive radioimmunoassay using goat anti-P28 antisera and ^{125}I P28. At the time of the last report we had isolated the P28 protein from the RD-114 virus and had succeeded in iodinating the P28 protein. We had also developed an antisera titration procedure in which a fixed concentration of antigen is titrated against increasing concentrations of antisera. The procedure is used for two purposes: 1) to determine optimal antibody concentration for use in the competitive radioimmunoassay, and 2) as a control measure to assess protein damage resulting from iodination of the P28 protein.

At this time, the P28 protein is being routinely iodinated, checked for iodination damage using the antisera titration procedure and used in the competitive radioimmunoassay procedure to determine P28 levels in feline cells which have been treated with the test chemicals.

2. Methodology

Treatment of FE cells (feline embryo) with carcinogens.

The FE cells are seeded into T75 flasks with McCoy's 5A medium with 10% fetal bovine serum (FBS). They are then incubated at 37°C for 24 hours after passage; the culture medium is then removed and replaced with 5 ml of McCoy's medium containing the carcinogen to be tested at a concentration determined by prior toxicity studies. The FE cells are then

incubated with the carcinogen for 30 minutes at 37°C. The carcinogen-containing media is then removed, the cells are rinsed with 10 ml of incomplete McCoy's and incubated in 30 ml McCoy's with 10% FBS. Cells are harvested at 24, 48 and 72 hours post-treatment. Control flasks (untreated) are also harvested at 24, 48 and 72 hours. Harvesting is accomplished as follows: the cells are rinsed twice with 10 ml of incomplete McCoy's, then treated with 3 ml of .25% trypsin. Cells are then suspended in 5 ml McCoy's containing 10% FBS and pelleted by centrifugation at 1000 rpm for 10 minutes. Supernatant is poured off and cells are resuspended in 5 ml of buffer (0.1M Tris HCl pH 7.8, 0.1 M NaCl, 0.05 M EDTA, 0.5% Triton X-100) and kept on ice. Then the cells are sonicated for 30 seconds and ultracentrifuged at 35,000 rpm for 60 minutes. The resulting supernatant is stored at -80°C and tested for the presence of P28 by the competitive radioimmunoassay and for total protein concentration by the Lowry modification of the Folin-Ciocalteau protein assay.

3 Lowry protein assay.

A solution is made up containing 50 ml 2% Na_2CO_3, 0.5 ml 1% $CuSO_4 \cdot 5H_2O$, and 0.5 ml 2% potassium tartrate (reagent A). Standard tubes are made up containing 20, 40, 80, 120, 160, and 200 µg, respectively, of bovine serum albumen in 5 ml reagent A. 0.5 ml of the solution to be tested is added to 5 ml of reagent A and all tubes are allowed to stand at room temperature for 10 minutes. 0.5 ml of 1 N phenol reagent is then added to each tube and after 30 minutes the color which develops is read at 750 nm in a spectrophotometer.

4 Iodination.

Purified P28 is radiolabeled by the chloramine-T method of Hunter and Greenwood.[6] Ten µg of P28 and 100 µg of chloramine-T are added to a

combi-vial containing 1 mCi of $Na^{125}I$. The contents of the vial are mixed for 60 seconds and the reaction is stopped by the addition of 250 µg of sodium metabisulfite. The iodination reaction is then quenched by the addition of 1 mg KI and .1 mg bovine serum albumen. The mixture is then chromatographed on a Biogel DG-G column (0.9 x 30 cm) to separate ^{125}I P28 from the free iodine (Fig. 1). The eluting buffer contains 0.01 M Tris HCl pH 7.8, 0.4 M NaCl, 0.3% Triton X-100, 0.1% NaN_3 and 1% bovine serum albumin. One ml fractions are collected and counted. A sample elution profile is attached. Two large peaks are seen; the first peak elutes on the void volume and contains the ^{125}I labeled P28 and the second contains the free iodine. Fractions from the preferred portion of the protein peak are pooled and diluted with elution buffer to a final concentration of 100 ng/ml, then stored at 4°C.

5 Titration of anti-P28 antisera vs. ^{125}I P28.

Procedures unchanged since last report with one exception: donkey anti-goat serum is used as secondary antibody instead of rabbit anti-goat serum in current research.

6 Competitive radioimmunoassay of P28 protein.

The competitive RIA is performed by the double antibody method. Reagents used are the following:

Antigen: P28 at 20 µg/ml (cold antigen)
^{125}I P28 at 1 ng/ml, specific activity, 25 µCi/µg (hot antigen)
Buffer: 0.01 M Tris HCl pH 7.8, 0.4 M NaCl, 0.3% Triton X-100, 0.1% NaN_3, 1% BSA

Primary antibody (1st Ab): goat anti-P28 antiserum
Buffer (used to dilute 1st Ab): 0.01 M Tris HCl pH 7.8, 0.03 M EDTA, 0.4 M NaCl, 0.3% Triton X-100, 0.1% NaN_3, 1% BSA, 1% normal goat serum

Secondary antibody (2nd Ab): donkey anti-goat antiserum
Buffer (used to reconstitute lyphilized 2nd Ab): 0.01 M Tris HCl pH 7.8, 0.001 M EDTA, 0.1 M NaCl, 0.1% Triton X-100

The RIA is run in four days. On day 1, serial two-fold dilutions of the cold antigen are made giving final concentrations ranging from 1.22 ng/ml up to 5000 ng/ml. 0.05 ml aliquots of each dilution are mixed with 0.05 ml of antiserum which has been diluted 1/500. 0.05 ml aliquots of each solution to be tested for P28 levels are also mixed with 0.05 ml of the antisera dilution. Control tubes are also set us as follows: Total tubes and background tubes contain 0.05 ml of antigen buffer and 0.05 ml 1st Ab buffer, reference tubes contain 0.05 ml antigen buffer and 0.05 ml 1st Ab. On day 2, 0.1 ml of hot antigen at 1 ng/ml is added to all tubes, and on day 3, 0.2 ml of undiluted 2nd Ab is added to all tubes except the total control tubes. On day 4, tubes are harvested by the following procedure: All tubes are diluted out to 1 ml by the addition of .78 ml of 2nd Ab buffer. The tubes are then centrifuged at 2500 rpm for 15 minutes, the supernatant is poured off and the pellets are counted. A standard curve is plotted by plotting antigen concentration against counts bound/counts in reference tubes and unknown P28 levels are calculated from the standard curve.

7. Experimental Results - Lowry protein assays on chemical-treated solubilized FE cells.

Chemicals:

Benzo(a)pyrene

	Total protein in micrograms		
	Trial #1	Trial #2	Ag
Day 1, control	265.99	200.00	232.995
Day 1, 5 ug/ml	235.79	276.08	255.94
Day 1, 15 µg/ml	238.80	336.39	287.595
Day 2, control	200.00	218.59	209.295
Day 2, 5 µg/ml	200.00	315.94	257.97
Day 3, 15 µg/ml	200.00	315.94	257.95
Day 3, control	205.94	295.85	297.42
Day 3, 5 ug/ml	200.00	513.81	356.90
Day 3, 15 µg/ml	305.78	537.95	421.86

Ø-B-naphthylamine

		Total protein in micrograms		
		Day 1	Day 2	Day 3
Trial #1	control	542.04	472.68	576.72
	5 µg/ml	542.04	937.39	542.04
	15 µg/ml	264.61	750.12	386.65
Trial #2	control	266.665	200.000	283.333
	5 µg/ml	249.997	200.000	212.466
	15 ug/ml	216.661	563.696	268.343
Trial #3	control	946.81	1430.39	949.48
	5 µg/ml	541.45	1394.84	612.57
	15 µg/ml	946.81	1131.71	776.13
mean average of 3 trials	control	585.17	701.02	616.51
	5 µg/ml	449.49	844.08	455.69
	15 µg/ml	476.03	815.18	471.04

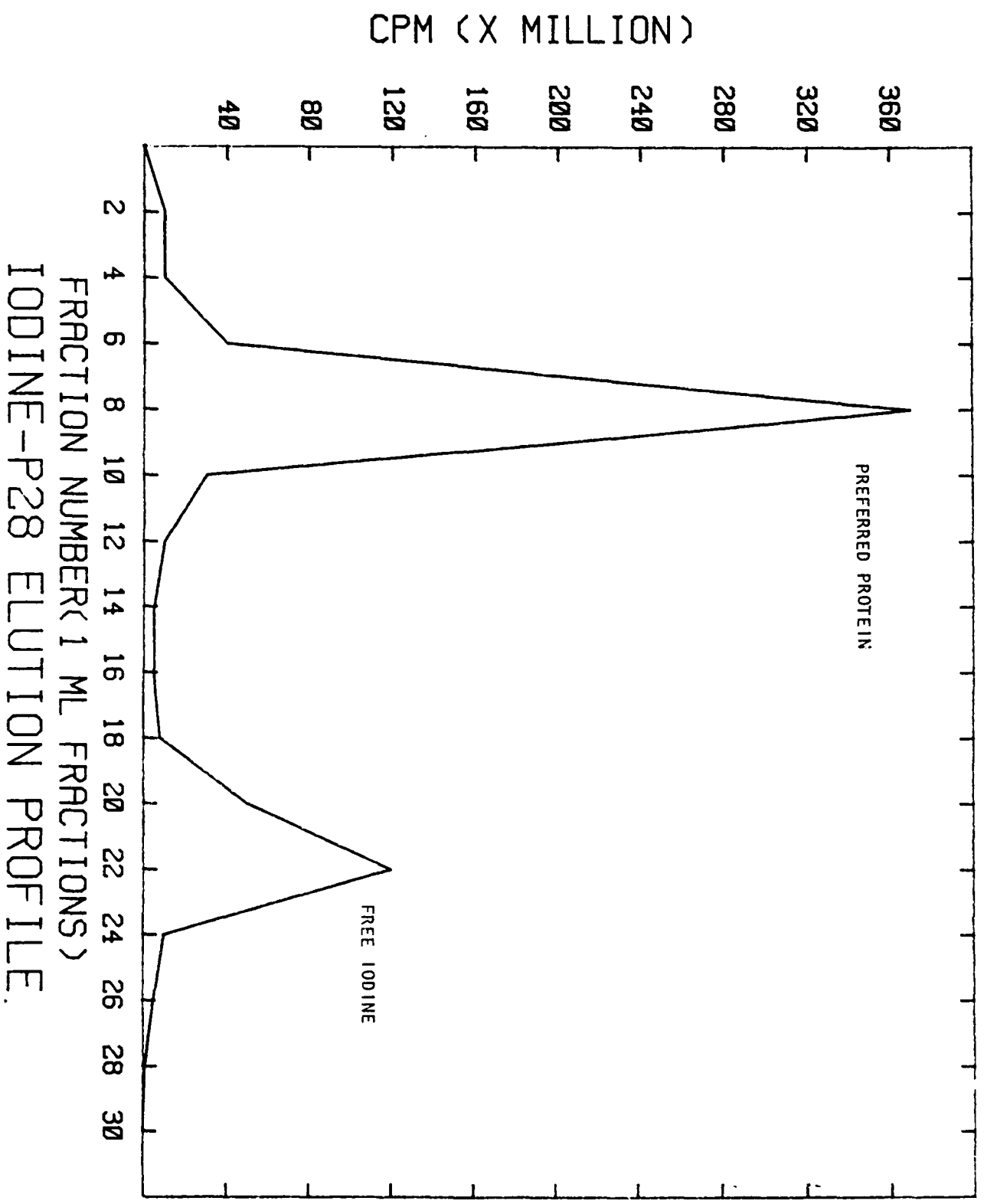


Fig. 1 - Chromatographic separation of iodinated RD-114 P28 fraction from free iodine.

Fig. 2 - RD-114 expression in FE cells treated with BP. □ = 15 µg/ml; X = 5 µg/ml; solid line = untreated control.

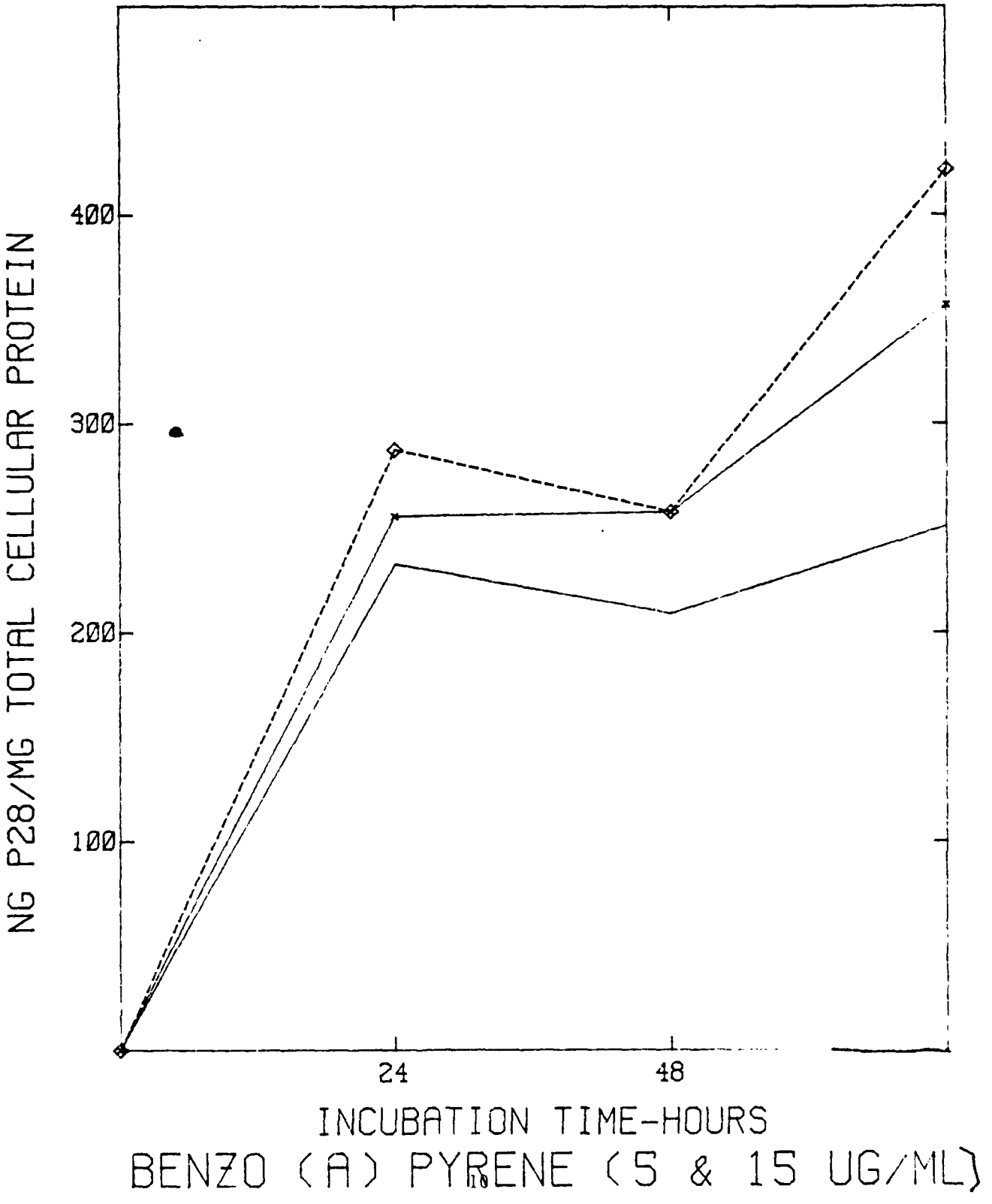
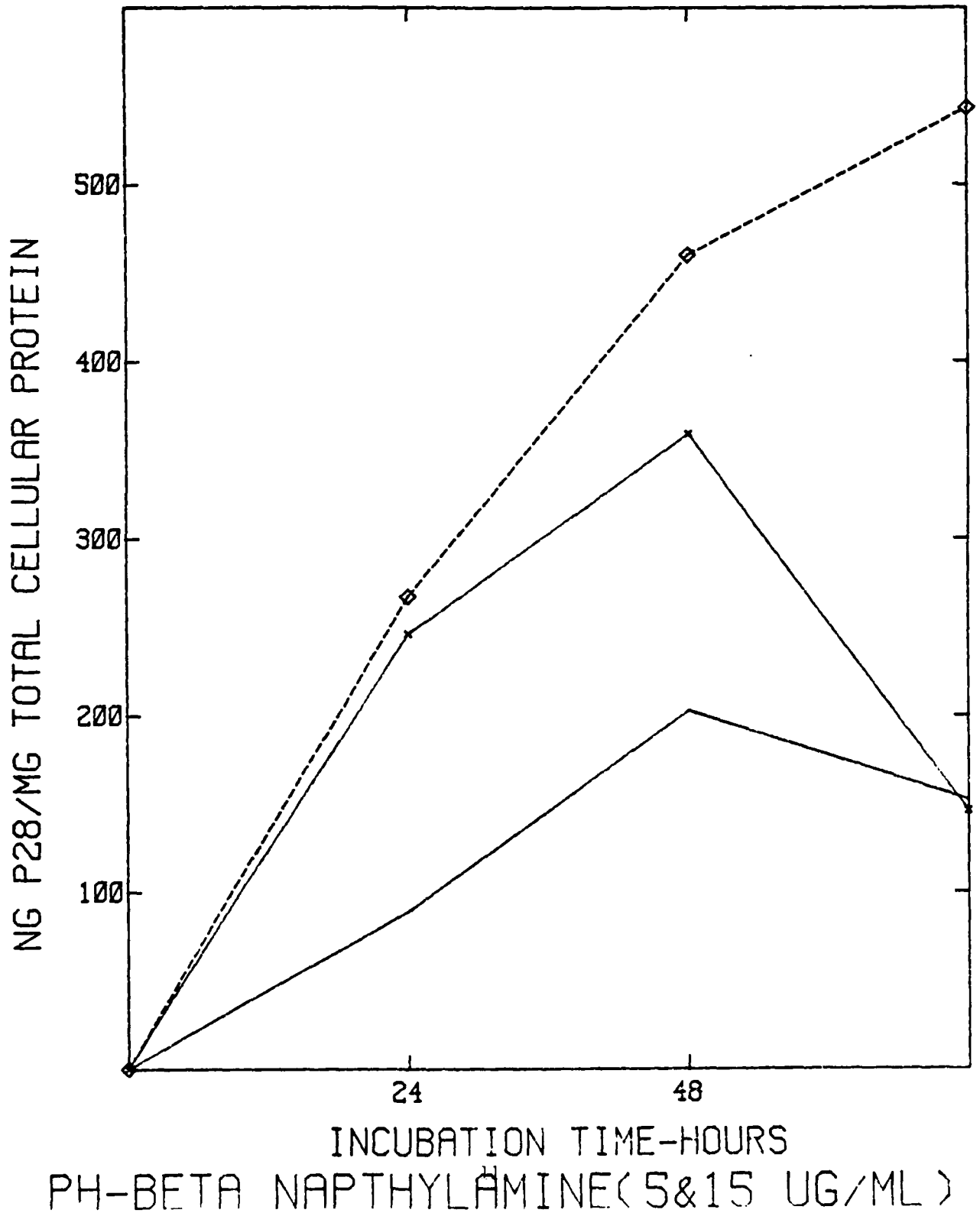


Fig. 3 - RD-114 expression in FE cells treated with phenyl-beta naphthylamine. □ = 15 µg/ml; X = 5 µg/ml; solid line = untreated control.



The results of BP and PBNA treatment on RD-114 expression are shown in Figs. 1 and 3. Cells were initially exposed to 5 or 15 µg/ml of chemical for 60 minutes. Cells were washed and incubated and plates for analysis pulled at 24, 48 and 72 hours. When stardardized as ng virus protein/mg total cellular protein, a time and dose-related effect was seen when compared to untreated control cells. BP treatment (Fig. 2) resulted in increased expression through and including 72 hours incubation, whereas with PBNA (Fig. 2), the low dose (5 µg/ml) increased in concentration through 48 hours and returned to control values at 72 hours, whereas the high dose (15 µg/ml) showed increased expression at all 3 time periods.

Passage of feline embryo cells in culture showed little effect on RD-114 P28 expression. As shown in Table 1, cells were examined through 16 passages. P28 protein concentrations ranged from .257 to 1.47 ng/mg total protein with a mean of 0.993 and a standard deviation of 0.390.

Table 1. RD-114 P28 Expression in Normal Feline Embryo Cells at Different Passage Levels

Passage Level[1]	Conc. RD-114 P28 ng/mg cellular protein	
1	.257	
2	N.t.[2]	
3	N.t.	
4	.422	
5	N.t.	
6	N.t.	
7	1.04	
8	1.03	
9	1.39	
10	1.18	
11	N.t.	
12	1.11	
13	1.20	mean = 0.993
14	N.t.	S.D. = 0.390
15	1.47	
16	.838	

1 = Passage level is defined as confluent monolayers trypsinized and split ratios of 1:3 used to propagate FE.
2 = N.t. = Not tested.

8. Molecular Virology.

Isolation of Feline Leukemia Virus RNA and Preparation of Complementary DNA.

Since January of 1982 much effort has been directed toward solving the problem of whether the culture conditions for growing the feline leukemia virus (FeLV-Kawakami Theilen strain) have contributed to degradation or nicking of the viral RNA. This has been a critical issue because intact RNA molecules or their subunits are required for uninterrupted copying of the genome into DNA. The size of the complementary DNA (C-DNA), made in an exogenous reaction using purified RNA and reverse transcriptase from Avian Myeloblastosis virus, has been used to determine whether there is a significant difference in the transcription of RNA isolated from virus grown under different culture regimes.

The reason for questioning the fidelity of the RNA isolated from virus grown under a 7 day culture regime was discussed in detail in the Continuation Proposal submitted to the Air Force Office of Scientific Research by the Ohio State University Research Foundation for July 1, 1982, desired starting date (Reference No. 14269-55-00), pages 12-16. The RNA was suspected of being nicked based on its sedimentation properties during centrifugation in sucrose density gradients after it was heated to dissociate it into its subunit structure. It was postulated that the extended growth period (7 days) in culture vs. a shorter culture regime might be contributing to increased chances for nuclease degradation. In efforts to verify this possibility, virus has been grown under drastically different culture conditions, but purified by procedures identical to those used previously and the isolated RNA has been compared by various parameters.

The seven day culture regime initially employed for production of FeLV for RNA isolation involved weekly seeding (day 1) of $1.2 - 1.4 \times 10^6$

FL-74 cells/ml into 200 ml of RPMI-1640 medium (GIBCO) plus 15% Newborn Calf serum in 0.5 gallon glass roller bottles. On day 5 these cultures were fed with an additional 200 ml of the same medium. The culture medium was harvested on day 8 and was either immediately clarified by centrifugation at 1200 rpm for 10 minutes to remove cells or was held at 4°C for periods of up to a week prior to clarification and virus purification.

Since January 1982 we began seeding the FL-74 cells at 2.5×10^6 cells/ml in seven liters of RPMI-1640 medium plus 15% newborn calf serum. These cells were gently suspended by means of slow stirring in a 15 liter glass Bellco Spinner Flask. The culture medium containing virus was harvested after only 12 hours of growth, and immediately clarified, with no intervening storage time at 4°C.

The procedures used for purifying the virus. FeLV and its RNA were identical for both culture times (7 day vs. 12 hour) and have been described previously (1). Virus purification essentially involved concentration at 4°C with a Millipore-Pellicon membrane filter unit; centrifugation at 25,000 rpm to further concentrate the virus; centrifugation in 20-50% linear sucrose density gradients; dialysis to remove sucrose; and final concentration by ultracentrifugation.

RNA was extracted from the FeLV using nuclease-free reagents and glassware. This involved an initial incubation with 1% SDS and Proteinase K at 500 ug/ml; 3 separate extractions with buffer saturated-phenol; precipitation at -20°C with absolute ethanol (2:1 v/v ratio); and purification by centrifugation in 15-30% sucrose density gradients at 38,000 rpm for 3.75 hours in the Beckman SW41 rotor.

The sedimentation profiles of the RNA obtained from each culture regime were compared. The characteristics of both native and heated RNA

from 7 day virus were described in detail in the Continuation Proposal submitted to the Air Force Office of Scientific Research by the Ohio State University Research Foundation for July 1, 1982, desired starting date (Reference No. 14269-55-00), pages 12-14. These profiles are again presented below for comparison to those obtained for RNA isolated from FeLV grown for 12 hours (see Fig. 4).

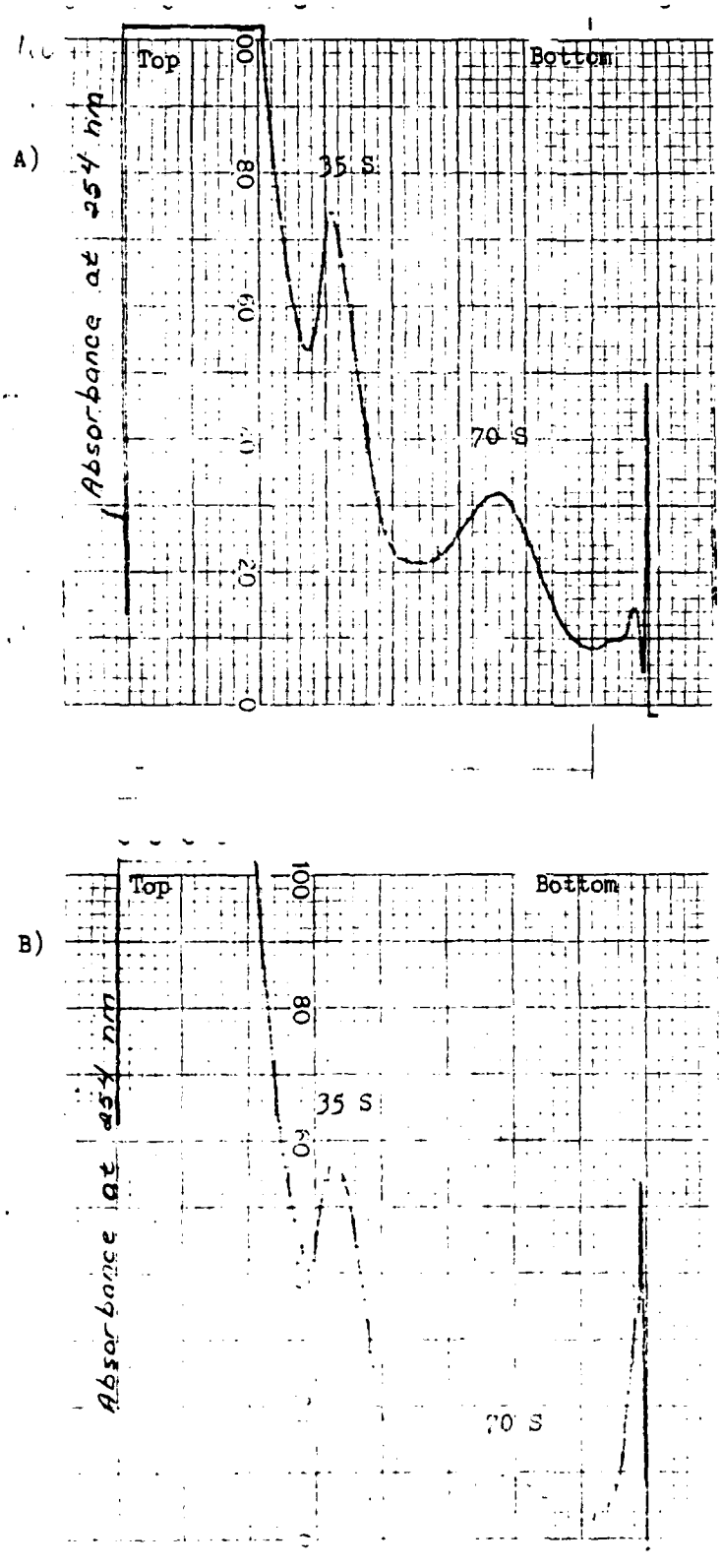


Figure 4

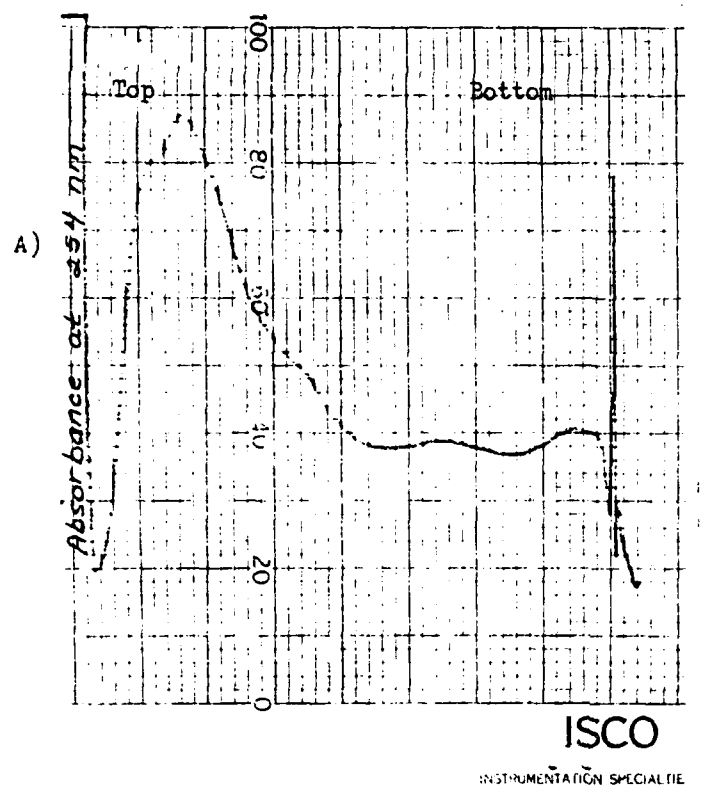
Profiles of the RNA from FeLV (KT strain) after centrifugation in 15-30% sucrose density gradients in TNE buffer (0.01 N Tris, 0.1 N NaCl, 0.001 M EDTA) plus 0.2% SDS. Centrifugation was for 3.75 hours in the Beckman SW41 rotor at 38,000 rpm and at 25°C.

Profile A represents RNA from FeLV grown for 7 days in culture.

Profile B represents RNA from FeLV grown for 12 hours in culture. Note somewhat lower yields obtained with the 12 hour culture conditions.

In both cases the native RNA appeared principally as two peaks sedimenting with values of approximately 35 S (subunit) and 70 S (dimer, full length viral RNA molecule) as determined with appropriate 16 S and 23 S marker RNAs from E. coli. In both cases there was a broad, highly absorbing (254 nm) region in the top one-third of the gradients due to 4 S t-RNA and smaller subunit RNA molecules. No substantial differences were noted in the character of the RNA profiles from virus grown for the two different time periods except that much lower yields were evident with the 12 hour harvest.

Of even greater interest was the similarity of sedimentation profiles of the 70 S RNA from 12 hour and 7 day harvests after heating in boiling water to dissociate it into its component subunits. We though that dissociation into the RNA subunits might increase the capability for longer length transcription of the viral genome. See Fig. 5 for profiles of the heated, dissociated 70 S RNA for both culture times.



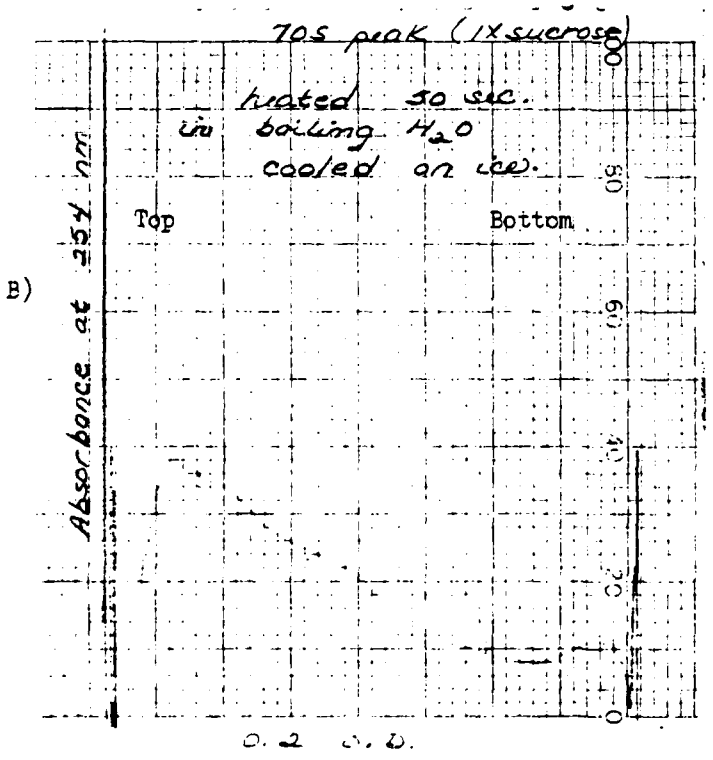


Figure 5

Profiles of the 70 S RNA from FeLV (KT strain) after thermal denaturation in boiling water for 45 seconds. The denatured 70 S RNA was centrifuged in 15-30% sucrose density gradients in TNE buffer plus 0.2% SDS. Centrifugation was in the Beckman SW41 rotor at 38,000 rpm and at 25°C for 3.75 hours.

Profile A represents heated 70 S RNA from virus grown for 7 days in culture.

Profile B represents heated 70 S RNA from virus grown for 12 hours in culture.

Because a majority of the heated 70 S RNA from the 7 day old virus sedimented in a broad band (5-25 S) near the top of the gradients rather than in the 35 S region, it had been concluded that the RNA was very probably nicked and held together by a large degree of secondary structure which was disrupted with heating.

When the 70 S RNA from virus grown for 12 hours was subjected to the same heating treatment, it surprisingly showed an identical sedimentation profile to that of the heated 70 S RNA from 7 day old virus. Because of the extreme precautions taken to avoid nuclease contamination and due to the reproducibility of the profiles of dissociated RNA, we began to question whether there truly was any significant degradation of the viral RNA. Such a dissociation profile may be characteristic for this particular virus strain (Kawakami-Theilen). It may possess low amounts of the 35 S subunit, but larger amounts of smaller heterogeneous subunits on dissociation. In concentrated preparations of RNA from 7 day old FeLV-KT we occasionally noted a distinct peak sedimenting at 16 S (Fig. 6). This may represent a characteristic subunit of FeLV-KT RNA involved in the assembly of the 70 S molecule.

Various RNA subunit sizes have been reported for several of the RNA retroviruses, all of which are thought to undergo assembly of their RNA into some form of dimer structure. After using thermal denaturation to reveal subunit classes of RNA, Kimble and Rea (19) found that three subunit classes existed in the Sneider-Theilen strain of feline sarcoma-leukemia virus (ST-FeSV-FeLV) RNA consisting of 35-37 S, 32-34 S, and 25 S.

Duesberg (4) has also reported on the melting behavior of certain RNA molecules of such RNA tumor viruses as Rous sarcoma virus (RSV) and mouse mammary tumor virus (MMTV). The melted RNAs all were

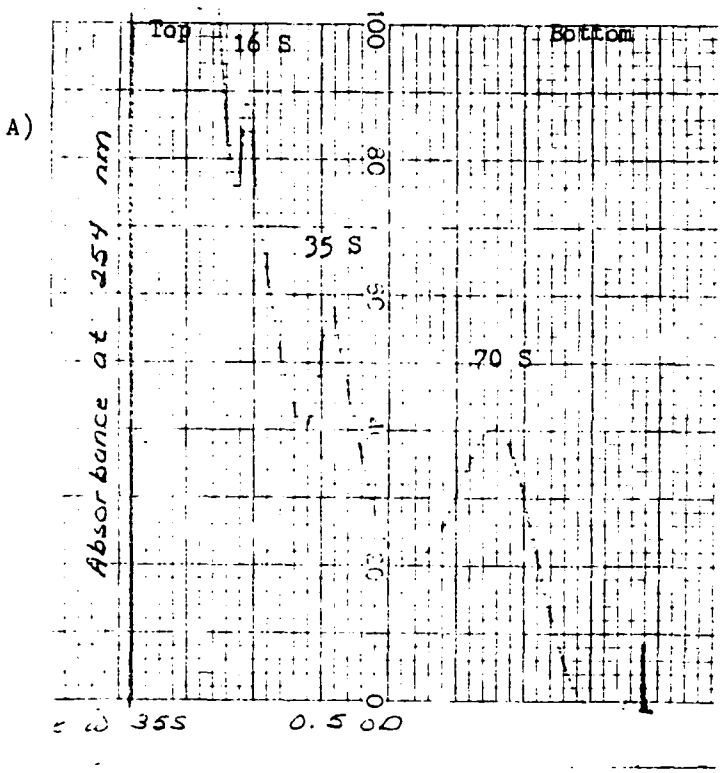
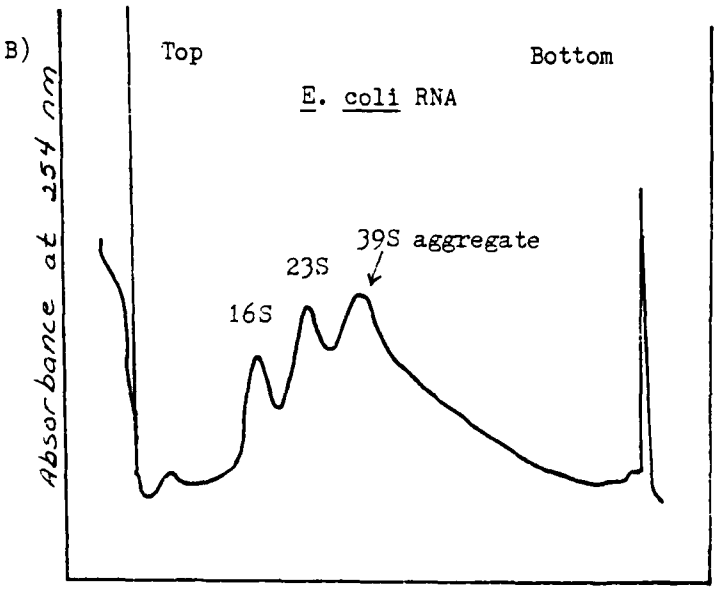


Figure 6

A) Profile occasionally seen of RNA from FeLV (KT strain) grown for 7 days, after centrifugation in 15-30% sucrose density gradients in TNE buffer (0.1 N Tris, 0.1 N NaCl, 0.002 M EDTA) plus 0.2% STS. Centrifugation was for 3.75 hours in the Beckman SW41 rotor at 38,000 rpm and at 25°C. Note peaks at 16 S, 35 S, and 70 S absorbing at 254 nm.

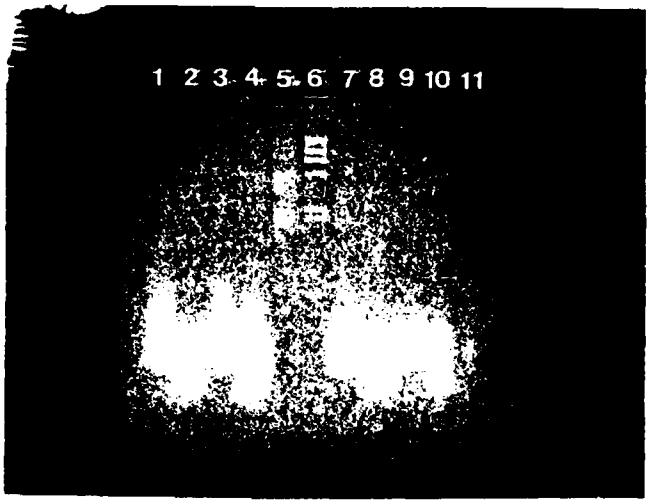


B) Profile of RNA markers from E. coli when subjected to the same conditions of centrifugation as described in Fig. 6A, above.

found to consist of a major component of about 3 x 10^6 molecular weight and a variable amount of heterogeneous smaller RNAs. Similar behavior has been noted for the RNAs of Rauscher murine leukemia virus (21), MMTV (5), and of the Schmidt-Ruppin and Bryan strains of RSV (12) in other studies, and also for the RNA of avian myeloblastosis virus (6).

We were unable to determine whether the RNA of FeLV-KT was truly nicked or whether heating dissociated the 70 S RNA into a heterogeneous mixture of smaller subunit RNAs. As a result, native 35 S and 70 S RNA isolated from FeLV grown for 12 hours or for 7 days in culture had been used to prepare complementary DNA. If the C-DNA of the 7 day old viral RNA was smaller in size than that synthesized from the 12 hour viral RNA, then we could assume that the 70 S RNA of the 7 day old virus was degraded and prevented transcription to the same extent. We have been sizing the C-DNA in 1.2% agarose gels according to the procedures of McMaster and Carmichael (11) (see Methods which follow). We are still attempting to determine a more exact size range of the C-DNAs with more accurate markers (Commercial Hind III and Eco RI digests of Lambda DNA have not been adequate for the size range in which we are working). We have found, however, that the C-DNAs obtained from both the 35 S and 70 S RNAs are the same size regardless of whether the virus has been grown for a short culture time (12 hours) or a much longer time (7 days). The glyoxal treatment used to denature the C-DNA for sizing in gels has provided evidence that the C-DNAs are in the expected size range (heterogeneous, with the majority predominantly less than 1000 bases) and show no evidence of nicking or degradation. They should serve as suitable probes in the hybridization reactions (Fig. 7).

Fig. 7



Complimentary DNA prepared to FeLV (KT strain) RNA after electrophoresis in a 1.2% agarose gel and 10 mM sodium phosphate buffer, pH 7.0 at a constant current of 45 mAMPs for 2 hours. The gel was stained with acridine orange at 15 ug/ml and destained overnight prior to being photographed.

C-DNA in tracks 1+2, 3+4, and 7+8 was electrophoresced prior to final purification on a Sephadex G-50 column.

Track 1 - C-DNA to 35 S RNA (12 hour old virus) denatured with glyoxal.
Track 2 - Nondenatured C-DNA to 35 S RNA (12 hour old virus).
Track 3 - C-DNA to 35 S RNA (7 day old virus) denatured with glyoxal.
Track 4 - Nondenatured C-DNA to 35 S RNA (7 day old virus).
Track 5 - Marker DNA - Hind III digest of Lambda DNA denatured with glyoxal. Bands are not evident due to nicking which is exposed on denaturation.
Track 6 - Nondenatured Marker DNA - Hind III digest of Lambda DNA. Bands occur at 23,606; 9,636; 6,636; 4,333; 2,257; 1,985; and 561 base pairs.
Track 7 - C-DNA to 70 S RNA (7 day old virus) denatured with glyoxal.
Track 8 - Nondenatured C-DNA to 70S RNA (7 day old virus).
Track 9 - C-DNA to 70 S RNA (7 day old virus) excluded from Sephadex as high molecular weight pure fraction - denatured with glyoxal.
Track 10- Same C-DNA as in Track 9, but nondenatured.
Track 11- Glyoxylated second fraction excluded from Sephadex containing non-reacted nucleotide bases and calf thymus primers from the exogenous reaction. These should not be evident on the gel due to their small size and rapid migration through the gel.

Note that all C-DNAs occur in the same size range and show virtually no degradation as evidenced by denaturation with glyoxal.

It appears that the RNA from FeLV-KT grown for 12 hours or for 7 days is very similar in structure and very probably intact. If the RNA is nicked, it must occur consistently in both 12 hour and 7 day old cultures and this is highly unlikely considering the precautions taken to prevent nuclease contamination (baking of glassware and diethyl pyrocarbonate treatment of all buffers and reagents).

II. Co-Carcinogenesis Treatments Resulting in the Transformation of Detroit$_{550}$ Cells Which Have Been Completed to Date are as Follows:

Compound	Concentration Administered	Time Administered Relative to FeLV-KT Infection
Hydrazine	60 ppm	2 hours prior 2 hours post
Methyl Hydrazine	100 ppm	2 hours prior 2 hours post
Unsymmetrical Dimethyl Hydrazine	100 ug/ml	2 hours prior 2 hours post
Symmetrical Dimethyl Hydrazine	100 ug/ml	2 hours prior 2 hours post
Benzo(a)pyrene	10 µg/ml	2 hours prior 2 hours post
Pyrene	10 µg/ml	2 hours prior 2 hours post

The Detroit$_{550}$ cells from the preceding experiments have been stored frozen at -80°C. The DNA will be extracted following preliminary treatment to isolate nuclear and cytoplasmic cellular fractions (see Methods section which follows).

a. Revised and New Methods

A) Revised Procedure for Preparation of Complementary DNA to 35 S or 70 S RNA of Feline Leukemia Virus (KT strain) in an Exogenous Reaction.

The following components are added in order to the final 1 ml reaction:

		Stock Concentration	Final Concentration
1)	Tris - HCl, pH 8.1 - 40 µl	500 mM	50 mM
2)	Double Distilled Deionized H_2O - 120 µl		
3)	$MgCl_2$ - 10 µl	800 mM in H_2O	8 mM
4)	d ATP d TTP d GTP 100 µl combined mixture	10 mM in 50 mM Tris-HCl, pH 8.1	1 mM each
5)	d CTP - 100 µl	10 mM in 50 mM Tris-HCl, pH 8.1	1 mM
6)	2-Mercaptoethanol - 10 µl	2 M in H_2O	20 mM
7)	FeLV RNA, 35 S or 70 S - 200 µl	250 µg/ml in 50 mM Tris-HCl, pH 8.1	50 µg/ml
8)	Calf Thymus Primers - 200 µl	2.5 mg/200 µl in 50 mM Tris-HCl,pH 8.1	2.5 mg/ml
9)	Bentonite - 80 µl	1 mg/ml in H_2O	80 µg/ml
10)	AMV Reverse Transcriptase - 40 µl	19,000 units/ml in Non-Tris buffer	750 units/ml
11)	KCl - 100 µl	1.4 M in H_2O	140 mM

Incubation is for 3 hours at 37°C in capped plastic microfuge tubes which have been siliconized and treated for nuclease contamination.

We have also been planning to prepare a labeled C-DNA probe using ^{35}S labeled d ATP. This would be of great advantage in that this probe would have a much longer half-life (87.2 days vs. 14.3 days) and a lower energy beta (0.167 MeV vs. 1.71 MeV) than a ^{32}P labeled C-DNA. In theory it should have a similar specific activity, and would allow for extended use in hybridization studies, eliminating the need for frequent syntheses of the short-lived ^{32}P C-DNA probe. If a ^{35}S labeled C-DNA does prove to be of too low a specific

activity for its use in our hybridization studies, then a ^{32}P labeled probe will be made by adding 2000-4000 μCi/ml of ^{32}P d CTP at 300-400 Ci/mmol specific activity to the C-DNA exogenous synthetic reaction in place of 1 mM unlabeled d CTP.

The procedures which are being used to terminate the C-DNA reaction are as follows:

1) Following the three hour incubation, an equal volume of a solution consisting of 100 mM EDTA, 1% SDS, and 1 mg Proteinase K/ml prepared in 50 mM Tris-HCl, pH 8.1 is added and the mixture is incubated for 30 minutes at 37°C.

2) This mixture is diluted to 5 ml with 50 mM Tris-HCl, pH 8.1 containing 0.1 M NaCl.

3) The 5 ml mixture is extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). The phenol is first saturated with 50 mM Tris-HCl, pH 8.1. Extraction is for 30 minutes at room temperature with gentle stirring. The mixture is then centrifuged at 2,000 rpm for 15 minutes and the upper aqueous phase is collected.

4) The aqueous phase is treated for 10 minutes at 65°C with 0.25 N NaOH to hydrolyze the template RNA. The mixture is then neutralized with HCl.

5) The aqueous phase containing the C-DNA is then placed at -20°C after adding 2 volumes of cold absolute ethanol and one-tenth volume of 2 M Na acetate to precipitate the C-DNA.

6) The single-stranded C-DNA is collected by centrifugation and separated from calf thymus primers and unincorporated nucleoside triphosphates by gel filtration on Sephadex G-50 using 50 mM Tris-HCl for elution at pH 8.1.

7) The pure C-DNA is precipitated again at -20°C with ethanol and Na acetate and is concentrated by centrifugation, resuspended in a small volume of 50 mM Tris-HCl, pH 8.1, and stored at -80°C.

B) Analysis of Complementary DNA Products on Agarose Gels for Size Determination.

These procedures are according to McMaster and Carmichael (11)

This method employs denaturation of nucleic acids and reaction with glyoxal, followed by electrophoresis in a slab gel system. Glyoxalation introduces an additional ring onto guanosine residues which sterically hinders the formation of G-C base pairs and, therefore, the renaturation of native structure, allowing for a much more accurate determination of molecular weight.

DNA samples are incubated in capped plastic microfuge tubes (nuclease-treated) and siliconized) for one hour at 50°C in 1.0 M glyoxal, 50% (v/v) dimethyl sulfoxide, and 10 mM NaH_2PO_4/Na_2HPO_4, pH 7.0. Control samples are treated identically but in the absence of glyoxal and Me_2 SO. Oxidation products are removed from glyoxal immediately prior to use by passing the glyoxal solution 4 times over a column of mixed bed ion-exchange resin AG-501-X8 (Bio-Rad).

After incubation, the samples are cooled to room temperature. Sucrose is added to 5% just before electrophoresis. The samples are electrophoresced in 1.2% agarose horizontal slab gels prepared in 10 mM NaH_2PO_4/Na_2HPO_4, pH 7.0, which also serves as the running buffer. Electrophoresis is at a constant current of 45 mAMPs for 2 hours at room temperature. Bromophenol blue at 0.25% in 50% glycerol serves as a marder dye. Currently we are using DNA molecular weight marders I and II, the Eco RI and Hind III digests of Lambda DNA (Boehringer Mannheim GmbH ' Biochemica).

C) Separation of Nuclear and Cytoplasmic Fractions of $Detroit_{550}$ Cells Treated with Chemicals and ST-FeSV-FeLV Prior to DNA Extraction.

These methods are based upon those of Ghosh et al., 1980 (8)

The cell pellet is suspended in hypotonic buffer consisting of 0.1 M Tris-HCl, pH 7.4; 0.01 M NaCl; and 0.003 M $MgCl_2$. The cells are allowed to swell for 20 minutes and lysed with 10 strokes in a tight fitting Dounce homogenizer followed by 4 passages through a 26-gauge hypodermic needle. This results in complete lysis of cells without apparent lysis of nuclei.

The cell lysate is centrifuged for 10 minutes at 4,000 rpm in a Sorval SS34 rotor. The cytoplasmic supernatant is decanted and saved. The nuclei are resuspended in hypotonic buffer and recentrifuged - the second cytoplasmic supernatant fraction is combined with the first.

The nuclear pellet is taken up in 10 ml of hypotonic buffer and a 1.5 ml mixture of 6.7% Tween 40 (v/v) and 3.3% Sodium deoxycholate (w/v) is added. This suspension is vortexed for 2-3 seconds and the nuclei are centrifuged a third time. A fourth resuspension in hypotonic buffer and resedimentation of the nuclei is done. This should free the nuclei of all traces of cytoplasmic material.

The cytoplasmic fractions will be combined and frozen at -80°C. If future study warrants, these cytoplasmic fractions will be treated with RNase and extracted for cytoplasmic DNA in efforts to detect levels of FeSV-FeLV C-DNA present in the cytoplasm prior to integration.

The nuclear fraction will be extracted for high molecular weight DNA, including integrated provirus (1).

D) Revised Procedures to be Used for DNA-DNA Hybridization.

Because of the limitations of the complementary-DNA synthetic reaction, the C-DNA which is made is single-stranded in nature. This presents certain problems in analyzing the reassociation kinetics of its hybridization with the

double-stranded DNA of the virus infected, and chemically treated $Detroit_{550}$ cells. For this reason, preliminary hybridization experiments will be done using controlled amounts of unlabeled C-DNA and ^{14}C-labeled $Detroit_{550}$ cells which have been infected with FeLV-FeSV-ST. This will determine the level of C-DNA which can be added to the reaction before the kinetics shift from a second order to a pseudo-first order reaction. It is necessary for our purposes to work under second order conditions. This situation can be maintained as long as the concentration of C-DNA added to the reaction is not in excess of the concentration of integrated provirus. The reassociation constants will be determined by analysis with a computer program based upon that developed by Britten et al. (3), and modified by Galau et al. (7), which involves statistical analysis by means of least squares. We are presently involved with adapting such a program for our own hybridization analyses.

Once the concentrations of cellular double-stranded DNA and single-stranded C-DNA necessary to maintain second-order kinetics are determined, these concentrations will be used throughout the hybridization analysis with DNA from the various chemical treatments. The quantity of integrated proviral sequnces will be calculated based upon changes in the reassociation constants relative to untreated $Detroit_{550}$ cells which have been infected with FeLV-FeSV-ST.

The stepwise procedures to be used for hybridization are as follows:

1) Stock concentrations of human cellular $Detroit_{550}$ DNA, radiolabeled C-DNA, and calf thymus DNA will be mixed in a 1 ml reaction volume and sonicated to reproducible lengths of 500-800 nucleotides. The standard concentrations of DNA to be used will be based on preliminary studies of the kinetics of reassociation using approximately 200 µg of $Detroit_{550}$ DNA, 1 ng (1000 cpm) of radiolabeled C-DNA, and calf thymus DNA to provide a constant total DNA concentration per 1 ml.

2) The sonicated DNA will be precipitated at -20°C with 2 volumes of cold, absolute ethanol, and one-tenth volume of Na acetate at 2.0 M.

3) THe precipitated DNA is resuspended in 400 µl of 2.5 mM EDTA and is further dialyzed against 2.5 mM EDTA. This is done to eliminate the possibility of concentrating salts with DNA during precipitation and thus to ensure that the temperature used for denaturation is well above the melting point of the DNA.

4) An equal volume of 2 X reassociation buffer consisting of 0.02 M Tris-HCl, pH 7.6, and 0.10% SDS is added and the mixture is heated for 10 minutes in boiling water to dissociate the strands. The denatured DNAs are quick-chilled in an ethanol-dry ice bath.

5) 200 µl of 3.25 M NaCl in 1 X reassociation buffer consisting of 0.01 M Tris-HCl, pH 7.6 and 0.05% SDS is added. The final 1 ml reaction mixture consists of 0.01 M Tris-HCl, pH 7.6; 0.05% SDS; and 1 mM EDTA according to the procedures used by Sherr et al. (13).

6) The 1 ml reaction mixture in capped mirofuge tubes is overlayed with sterile mineral oil and incubated at 67°C for up to 5 days. Aliquots of 0.1 ml will be withdrawn at regular intervals and frozen at -8-°C.

7) All sample aliquots from a given reassociation experiment will be thawed and diluted at the same time in S_1 nuclease buffer consisting of 0.25 M NaCl; 1 mM Zn acetate; and 0.03 M Na acetate, pH 4.5. A 20-45 µl sample of the diluted mixture is immediately applied to a 2.4 cm Whatman DE-81 filter disk to determine the total radioactivity per unit volume.

8) S_1 nuclease is added to the remaining volume to a final concentration of 5,000-10,000 units/ml and this mixture is incubated for 80 minutes at 37°C. S_1 nuclease digestion is based on the procedures of Maxwell et al. (10).

9) After incubation, a 45-75 µl sample is withdrawn and applied to a DE-81 filter disk for determination of the radioactivity in reassociated DNA.

10) The filters are washed in 3-4 changes of 0.5 M phosphate buffer, followed by 2 separate washes in H_2O, and a final wash in 95% ethanol prior to being thoroughly dried.

11) Finally, the filters are counted in 5 ml of a PPO-POPOP, toluene scintillation mixture in 20 ml glass scintillation vials. The unused samples and filter disks will be stored in low density, thick-walled containers long enough for adequate decomposition prior to disposal.

REFERENCES

1. The Ohio State University Research Foundation - Annual Report Prepared for the Air Force Office of Scientific Research for the period July 31, 1980 - September 15, 1981. Contract No. F49620-80-C-0087. Molecular Interactions of High Energy Fuels and Jet Fuels with Oncogenic Viruses and Endogenous Viruses.
2. Blair, C.D. and Duesberg, P.H. 1968. Structure of Rauscher Mouse Leukemia Virus DNA. Nature (London) 220: 396-399.
3. Britten, R.J., Graham, D.E., and Neufeld, B.R. 1974. Analysis of Repeating DNA Sequences by Reassociation In Methods in Enzymology, Ed. Grossman, L. and Moldave, K., Academic Press, New York, New York, Vol. 29, P. 363-418.
4. Duesberg, P.H. 1970. On the Structure of RNA Tumor Viruses. Current Topics in Microbiology and Immunology. 51: 79-104.
5. Duesberg, P.H., and Cardiff, R.D. 1968. Structural Relationships Between the RNA of Mammary Tumor Virus and Those of Other RNA Tumor Viruses. Virology 36: 696-700.
6. Erikson, R.L. 1969. Studies on the RNA from Avian Myeloblastosis Virus. Virology 37: 124-131.
7. Galau, G.A., Britten, R.J., and Davidson, E.H. 1977. Studies on Nucleic Acid Reassociation Kinetics: Rate of Hybridization of Excess RNA with DNA, Compared to the Rate of DNA Renaturation. Proc. Natl. Acad. Sci. USA Vol 74, No. 3 P. 1020-1023.
8. Ghosh, P.K., Reddy, V.B., Piatak, M., Lebowitz, P., and Weissman, S.M. 1980. Determination of RNA Sequences by Primer Directed Synthesis and Sequencing of Their C-DNA Transcripts. In Methods in Enzymology, Ed. Grossman, L., and Moldave, K. Vol. 65, Nucleac Acids P. 580-595.

9. Kimble, P.C., and Rea, T.J. 1980. Three Sizes of Subunits in RNAs from Feline Sarcoma-Leukaemia Virus Mixtures. J. Gen. Virology 51: 295-305.

10. Maxwell, I.H., Van Ness, J., and Hahn, W.E. 1978. Assay of DNA-RNA Hybrids by S_1 Nuclease Digestion and Adsorption of DEAE-Cellulose Filters. Nucleic Acids Research Vol. 5 (No. 6): 2033-2038.

11. McMaster, G.K. and Carmichael, G.C. 1977. Analysis of Single- and Double-Stranded Nucleic Acids on Polyacrylamide and Agarose Gels by Using Glyoxal and Acridine Orange. Proc. Natl. Acad. Sci. USA Vol. 74, No. 11, P. 4835-4838.

12. Montagnier, L., Golde, A., and Vigier, P. 1969. A Possible Subunit Structure of Rous Sarcoma Virus RNA. J. Gen. Virol. 4: 449-452.

13. Sherr, C.J., Fedele, L.A., Oskarsson, M., Maizel, J., and Vands Woude, G. 1980. Molecular Cloning of Snyder-Theilen, Feline Leukemia and Sarcoma Viruses: Comparative Studies of Feline Sarcoma Virus with its Natural Helper Virus and with Moloney Murine Sarcoma Virus. J. Virology 34 (No. 1): 200-212.

10. FeSV-Co-Carcinogenesis Assays

Table 1. Toxicity Assays of Chemicals to be Tested in FeSV Assay

Chemicals	Concentration (µg/ml) Percent Cell Survival[1]						
	0.001	0.01	0.1	1.0	10.0	100	1000
Phenanthracene	N.T.	71	70	90	86	3.4	0
Anthracene	N.T.	91	89	94	100	95	4
2 Aminobyphenyl	81	94	91	82	100	91	N.T.
4 Aminobyphenyl	100	100	100	100	100	75	N.T.

1. Determined by: $100\% - \left(\frac{\text{\# Clones [treated]}}{\text{\# Clones [control]}}\right) X\ 100$

N.T. = Not Tested.

Chemicals assayed to date and the correlation of virus inhibition and reported carcinogenic activity are shown in Table 2.

Table 2. Correlation Between Inhibition of Virus Transformation and Diverse Chemicals Inducing Tumors in Rodents.

	Chemical Group	Reported Activity[1]	Inhibition of ST-FeSV Transformation
I.	Aromatic Amines		
	A. Naphthyl Amines		
	1. Two (2)	C	Yes
	2. Phenyl-alpha	C	Yes
	3. Phenyl-beta	C	Yes
	B. N-Acetoxy 2 fluorenyl-acetamide	C	Yes
II.	Polycyclic Hydrocarbons		
	1. Benzo(a)pyrene	C	Yes
	2. Pyrene	NC	No
III.	Hydrazines		
	1. Hydrazine	C	Yes
	2. Mono-methyl hydrazine	C	Yes
	3. 1,1 dimethyl hydrazine	C	Yes
	4. 1,2 dimethyl hydrazine	C	Yes
IV.	Other		
	1. Aflatoxin B_1	C	Yes
	2. *Amosite* Asbestos	C	Yes
	3. JP5 (Shale)	NC	No
	4. JP5 (Petrol)	NC	No
	5. RJ5 (TH dimer)	NC	No
	6. Diesel fuel, Marine	NC	No
	7. Acetone	NC	No
	8. Methyl Azoxymethanol-acetate	C	Yes
	9. Formalin	C	Yes
	10. Triton X-100	?	No

1. C = carcinogen; NC = non-carcinogen.

From: (1) Advances in Modern Environmental Toxicology Vol. I. Eds, N. Mishra, V. Dunkel, M. Mehlman. Senate Press, N.J. 1980.

(2) "Origins of Human Cancer", by I.J. Selikoff, Eds. H. Hiatt, J.D. Watson, and J.A. Winsten. Cold Spring Harbor, N.Y. 1977.

(3) Unpublished data from Toxicology Branch, AMRL, WPAFB.

11. Double-Blind Study

These studies are proceeding and, at this time, 8 chemicals have been tested for toxicity and evaluated in co-carcinogenesis assay. Until all 24 chemicals have been evaluated, the results of the double-blind study will not be known.

III. Written Publications

1. Blakeslee, J.R., Elliot, A.M., and Carter, L.J. (1982) In Vitro Effects of Polynuclear Aromatic Hydrocarbon on FeSV Transformation of Human Cells. In Press: Polynuclear Aromatic Hydrocarbons: Combustion Processes, Environmental Effects, and Biochemistry, 7th International Symposium.

2. Blakeslee, J.R., Elliot, A.M., and Carter, L.J. (1982) Anti-Oncogenic Activity of Diverse Chemical Carcinogens on Retrovirus-Induced Transformation of Human Skin Fibroblasts. In Press: Annals of N.Y. Academy of Science Conference on Cellular Systems for Toxicity Testing.

IV. Oral Presentations

1. Seminar - Department of Veterinary Preventive Medicine, Ohio State University, March 10, 1982.

2. N.Y. Academy of Science Conference on Cellular Systems for Toxicity. Barbizon Plaza Hotel, October 5, 1982.

3. Seventh International Symposium on Polynuclear Aromatic Hydrocarbons. Battelle Memorial Institute, Columbus, Ohio, October 27, 1982.

4. Seminar - Department of Radiology (Chemical, Biological, Radiological Experimental Research Group, School of Medicine, The Ohio State University, November 5, 1982.

5. Seminar - Department of Veterinary Pathobiology, The Ohio State University, January 2, 1983.

V. Professional Personnel Associated with Research Effort

A. James R. Blakeslee, Jr., Ph.D.

Department of Veterinary Pathobiology

The Ohio State University

Columbus, Ohio 43210

Ann Elliot, M.Sc.

Department of Veterinary Pathobiology

Wendy G. Souder, B.Sc.

Department of Veterinary Pathobiology

B. Collaborators

David Longfellow, Ph.D.

Assistant Chief

Chemical and Physical Carcinogenesis Branch

Division of Cancer Cause and Prevention

NCI, NIH,

Bethesda, MD 20205

VI. Significance of Results to Date and Future Direction

The significance of this research, if validated by the double-blind study, will provide a short-term predictable assay for determination of carcinogenic potential of new and existing chemicals. This type of assay would permit a more selective process in validation of toxicity and carcinogenicity studies in rodent assays. Now required, the hybridization and endogenous virus studies will provide evidence of chemical effect on genetic expression, control and translational products. These studies will help elucidate molecular mechanisms involved in transformation of normal cells to the neoplastic state.

VII. No Animals Were Used in This Study

VIII. Appendix

7th International Symposium on Polynuclear Aromatic Hydrocarbons,
Battelle Memorial Institute, October 26-28, 1982. Columbus, OH. USA.

In Vitro Effects of Polynuclear Aromatic Hydrocarbons on FeSV Transformation of HumanCells

J. Blakeslee,[1,2,3] A. Elliott[1] and L. Carter[1]
Department of Veterinary Pathobiology[1], Department of Microbiology[2]
and the OSU Comprehensive Cancer Research Center[3]

Previous studies from this laboratory described the inhibition of feline sarcoma virus transformation of human skin fibroblast cells by the chemical carcinogens, benzo(a)pyrene (BP), n-acetoxy 2-fluorenylacetamide (A-AAF) or aflatoxin B1 (AFB1), all of which significantly inhibited in vitro feline sarcoma virus transformation (Chem. Biol. Interact 23:1-11, 1978). The inhibition of transformation resulting from chemical treatment was not related to cytotoxic concentrations of carcinogens in that little or no effect on cells surviving treatment was noted (vide supra). Continued studies showed that the carcinogens inhibited a specific-viral gene function, i.e. transformation, but not other viral gene products which were shown to be cell-mediated (Comparative Leukemia Res. 1979, pp. 87-88, Elsevier, 1980). The studies reported here expanded the classes of polynuclear aromatic hydrocarbons tested to include jet fuels and aromatic amines used as lubricant additives - alpha and beta-naphthylamine and phenyl-alpha and phenyl-beta naphthylamine.

In vitro toxicity results show no significant difference in toxicity between shale oil derived (SOD) or petroleum derived (PD) fuels. LD_{50}s (ppm) for SOD-JP5 and PD-JP5 were 102 and 100, respectively, SOD-diesel fuel, marine (DFM) and PD-DFM were 87 and 85. LD_{50}s for JP10 and RJ5 were 91 and 19. No inhibition of FeSV transformation resulted with any of the fuels tested to date.

However, the napthylamines significantly inhibited virus transformation over a wide time frame - ranging from 24 hours before infection to 24 hours after infection. In most instances, the degree of suppression showed a good dose-relationship.

The data compiled from the FeSV transformation assay correlated well with the carcinogenic potential as determined by rodent tumor assays (Adv. in Modern Toxic, Eds. N. Mishra, V. Dunkel, and M. Mehlman, Senate Press, N.J., 1980) and in vitro chemical transformation assays in human cells (Proc. IX Conf. on Env. Toxic. AMRL-TR-79-68, 1979).

Supported by Air Force Office of Scientific Research Contract F49620-80-C-0087

In Press, 1982 - Annals of NY Acad Sci - Cellular Systems for Toxicity Testing

Anti-Oncogenic Activity of Diverse Chemical Carcinogens on Retrovirus-Induced Transformation of Human Skin Fibroblasts

J.R. Blakeslee,[1,2] A.M. Elliott[1] and L.J. Carter.[1] Department of Veterinary Pathobiology,[1] Department of Microbiology[2] and The Ohio State University Comprehensive Cancer Center,[2] 1925 Coffey Rd., Columbus, OH 43210.

Introduction

The utilization of a rapid in vitro assay to evaluate chemicals for carcinogenic activity would greatly reduce the time now required to determine carcinogenicity in animal test systems. The objectives of these studies were to determine whether chemicals altered ST-FeSV virus-directed transformation of human skin fibroblasts in a predictable manner and to correlate the alteration with carcinogenic or non-carcinogenic activity of the test chemical. The procedures used to study these interactions were previously described (1,2).

The results, to date, showed that diverse classes of carcinogens inhibited virus transformation when virus-infected cells were exposed to test chemical 2 hrs post-infection (p.i.) while non-carcinogens had no significant effect on transformation at 2 hrs p.i. Continued studies showed that the carcinogens inhibited a specific-viral gene function, i.e., transformation, but not other viral gene products (FOCMA, GSA and reverse transcriptase) which were shown to be cell-mediated. The studies reported here expanded the classes of carcinogens and non-carcinogens tested to include hydrazines, jet fuels, asbestos, formalin, methylazoxy methanol acetate and aromatic amines used as lubricant additives - alpha and beta naphthylamine and phenyl alpha and phenyl beta naphthylamine.

Materials and Methods

Cells and Virus - Detroit 550, normal ♂ skin fibroblasts (HSF) were grown under standard conditions as previously described (1). The Snyder-Theilen strain of feline sarcoma virus was prepared from feline embryo cell culture, titrated in HSF, and stored at -85°C.

Transformation Assays - Preconfluent log phase growth HSF were seeded into 16mm diameter wells at $4X10^4$ cells/well in 1.5 ml complete medium and incubated 18 hr prior to treatment in 5% CO_2 at 37°C. Cells were infected with 20 focus-forming unts/well. Cells chemically treated prior to virus infection were exposed to chemicals for 30 min., washed and refed until virus infection. Virus-infected infected cells were treated 30 min. with chemicals, washed and refed with complete medium. Seven-10 days p.i., cells were fixed and stained with Giemsa (1) and foci enumerated with a 25-40X dissecting microscope. Data evaluated statistically by the Student "t" test.

Results

The data displayed in Fig. 1 is typical of the results for chemical-treated virus-infected cells. Pre-determined non-toxic doses of commercial formalin (37% HCHO-10% CH_3OH) at 10 ppm and 1 ppm were used. Ten ppm significantly inhibited virus transformation by values ranging from 50% to 90% of control values while 1 ppm affected virus transformation to a lesser degree. Although transformation was inhibited at 4 time periods for formalin, the results of our studies with over 20 different chemicals (Table 1), show the time period(s) most affected are 2 or 6 hr post-infection. The results shown in Table 1 are based on these time periods. The data compiled from this assay correlated well with the carcinogenic potential determined by the standard rodent assays.

References

1. Blakeslee, J.R. and Milo, G.E. Chem.-Biol. Interactions, 23:1-11, 1978.
2. Blakeslee, J.R. AGARD Conf. Proceed. 309, pp. B6, 1-6, 1981.
3. Blakeslee, J.R., Elliot, A.M. and Turner, D.G. Adv. in Comp. Leuk. Res., 1979, Eds. D.S. Yohn, B.A. Lapin and J.R. Blakeslee, Elsevier, N. Holland, N.Y. pp. 87-88, 1980.
4. Fiala, E. Cancer 40:2436-2445, 1977.

Supported by AFOSR Contract F49620-80-C-0087.

Table 1. Correlation Between Inhibition of Virus Transformation and Diverse Chemicals Inducing Tumors in Rodents.

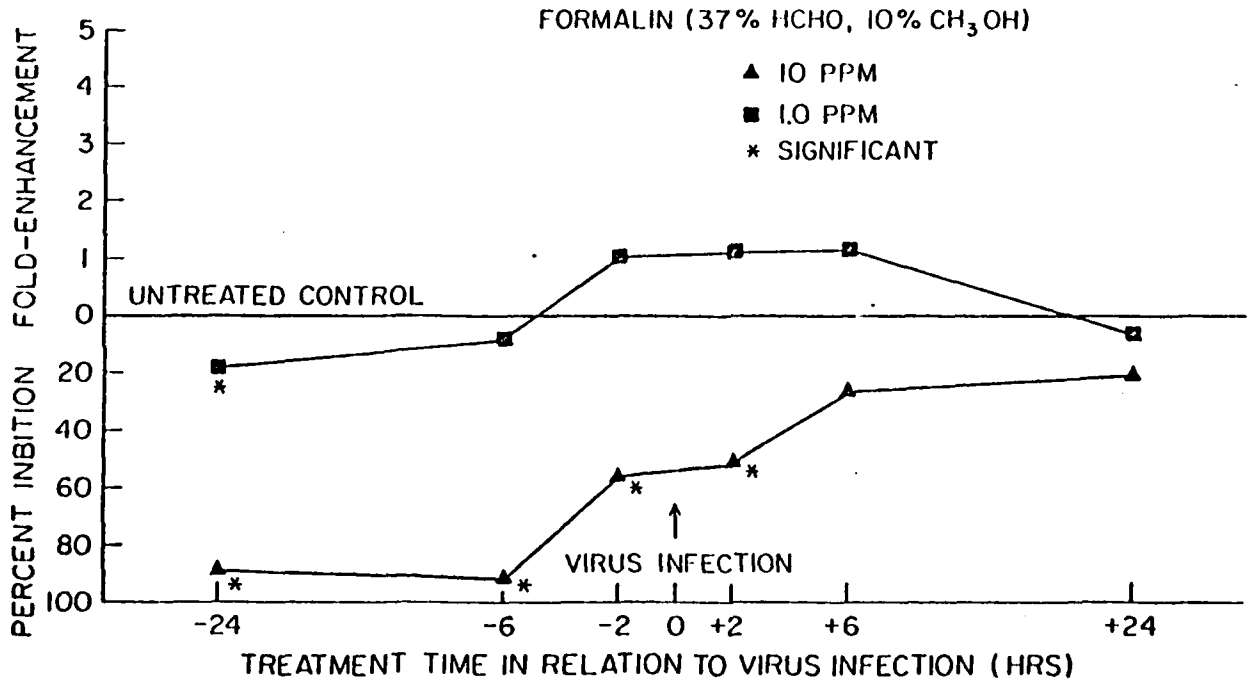
	Chemical Group	Reported Activity[1]	Inhibition of ST-FeSV Transformation
I.	Aromatic Amines		
A.	Naphthyl Amines		
1.	Two (2)	C	Yes
2.	Phenyl-alpha	C	Yes
3.	Phenyl-beta	C	Yes
4.	N-Acetoxy 2 fluorenyl-acetamide	C	Yes
II.	Polycyclic Hydrocarbons		
1.	Benzo(a)pyrene	C	Yes
2.	Pyrene	NC	No
III.	Hydrazines		
1.	Hydrazine	C	Yes
2.	Mono-methyl hydrazine	C	Yes
3.	1,1 dimethyl hydrazine	C	Yes
4.	1,2 dimethyl hydrazine	C	Yes
IV.	Other		
1.	Aflatoxin B_1	C	Yes
2.	Amosite Asbestos	C	Yes
3.	JP5 (Shale)	NC	No
4.	JP5 (Petrol)	NC	No
5.	RJ5 (TH dimer)	NC	No
6.	Diesel fuel, Marine	NC	No
7.	Acetone	NC	No
8.	Methyl Azoxymethanol-acetate	C	Yes
9.	Formalin	C	Yes
10.	Triton X-100	?	No

1. C = carcinogen; NC = non-carcinogen.

From: (1) Advances in Modern Environmental Toxicology Vol. I. Eds, N. Mishra, V. Dunkel, M. Mehlman. Senate Press, N.J. 1980.
(2) "Origins of Human Cancer", by I.J. Selikoff, Eds. H. Hiatt, J.D. Watson, and J.A. Winsten. Cold Spring Harbor, N.Y. 1977.
(3) Unpublished data from Toxicology Branch, AMRL, WPAFB.

Figure 1.

Commercial formalin (37% HCHO - 10% CH_3OH) was diluted in CM at 10 ppm and 1 ppm final concentration. Cells were treated and infected as described in Materials and Methods. (p) - Student's "t" test.


FORMALIN (37% HCHO, 10% CH_3OH)
▲ 10 PPM
■ 1.0 PPM
* SIGNIFICANT
PERCENT INBITION FOLD-ENHANCEMENT
5
4
3
2
1
0
20
40
60
80
100
UNTREATED CONTROL
VIRUS INFECTION
-24
-6
-2
0
+2
+6
+24
TREATMENT TIME IN RELATION TO VIRUS INFECTION (HRS)

IN VITRO EFFECTS OF POLYNUCLEAR AROMATIC HYDROCARBONS ON FeSV TRANSFORMATION OF HUMAN CELLS.

JAMES R. BLAKESLEE[1,2,3], ANN M. ELLIOT[1], LINDA J. CARTER[1]
Department of Veterinary Pathobiology[1], Department of Microbiology[2], and the OSU Comprehensive Cancer Center[3], 1925 Coffey Rd., Columbus, OH 43210, USA.

INTRODUCTION

A rapid in vitro assay to evaluate chemicals for carcinogenic activity would greatly reduce the time now required with animal test systems. Therefore, studies were undertaken to determine whether chemicals altered Snyder-Theilen feline sarcoma virus (ST-FeSV) directed transformation of human skin fibroblasts (HSF) in a predictable manner and to correlate the alteration with carcinogenic or non-carcinogenic activity of the test chemical. We initially reported that Benzo(a)-pyrene (BaP), N-acetoxy 2-fluorenylacetamide (A-AAF) or aflatoxin B1 (AFB1), inhibited virus-directed ST-FeSV transformation of HSF (1). It was further shown that the carcinogen treatment inhibited a specific virus gene function, i.e. transformation, but not virus synthesis. Other viral gene products, RNA-dependent DNA polymerase (RDDP), Group-Specific Antigens (GSA) and Feline Oncornavirus-Associated Cell Membrane Antigen (FOCMA) were detected in both carcinogen-treated or non-treated virus-infected cells (2). These studies suggested the inhibitory effect on virus-directed transformation was mediated by the carcinogen treatment, while the inhibitory effect on complete virus synthesis was cell-mediated. Further, the inhibitory effect of the carcinogens was abrogated when chemicals were added to virus-infected cells 48 hours post-infection (p.i.). A time-dependent function was also observed which showed diverse classes of carcinogens inhibited virus transformation when virus-infected cells were exposed to test chemicals 2 to 6 hours post-infection, while non-carcinogenic chemicals had no significant effect on transformation (3).

As a continuation of these studies we correlated the effect of the following chemicals with ST-FeSV transformation:

1) 2-naphthylamine (NA)
2) phenyl alpha naphthylamine (PANA)
3) phenyl beta naphthylamine (PBNA)
4) petroleum-derived JP5 jet fuel (PD-JP5)
5) shale oil-derived JP5 jet fuel (SD-JP5)

In Press - Proceedings 7th International Symposium on Polynuclear Aromatic Hydrocarbons.

6) 1,2 symmetrical dimethyl hydrazine (SDMH)
7) methyl azoxymethanol (acetate) (MAMA)
8) tetraethylthiuram disulfide (disulferam [DS])

MATERIALS AND METHODS

Cells

Human foreskin fibroblast cells (Detroit 550-CCL-109, American Type Culture Collection, Rockville, MD) were grown in Minimal Essential Medium with Earles salts supplemented with 1.0 mM sodium pyruvate, 2 mM glutamine, 1% non-essential amino acids, 50 µg/ml gentamycin (Schering Diagnostics, Port Reading, NJ) 0.22% sodium bicarbonate and 10% fetal bovine serum (Sterile Systems, Logan UT), thereafter designated Complete Medium (CM). Cells were serially passaged every 3-4 days at 1:2 split ratios and incubated at 37C in 5% CO_2.

Virus

The preparation of stock ST-FeSV was described previously (4). Briefly, 10% cell-free tumor homogenates were prepared and frozen at -85C in L15 medium and 5% FBS.

Transformation

Preconfluent log phase growth HSF cells were trypsinized and 4×10^4 cells seeded onto 16 mm wells (Costar, Cambridge, MA) in 1.0 ml CM and incubated 18 hrs. prior to treatment. Cells pre-treated with chemicals prior to virus infection were incubated 90 min. with appropriate chemical concentration at 2, 6, or 24 hr. pre-infection. Cells were washed 2X in CM, refed and incubated at 37C until virus infection. Cells to be infected were washed with serum-free CM and treated with 0.2 ml of DEAE-Dextran (40 µg/ml Sigma, St. Louis, MO]) in serum-free CM. After 20 min., the cells were washed with CM + 5% FBS, infected with 0.05 ml ST-FeSV, diluted to 1,000 focus-forming units (FFU) per ml. Twelve wells were used for each time period. Plates were rocked at 10-15 min. intervals and virus adsorbed 2 hr. at 37C. After adsorption, the inocula were aspirated and cells refed with 2.0 ml CM. Virus-infected cells were treated at 2, 6, or 24 hr. following virus adsorption by incubating infected cells with designated concentration of chemical for 90 min. followed by washing and refeeding cells with 2.0 ml CM. The cells were refed with CM on the 6th day p.i., and subse-

fixed with 10% phosphate-buffered formalin and stained with Giemsa 3-4 days later. Foci were counted at 25-40X magnification with a dissecting microscope in non-treated (control) and chemically-treated wells. FFU ± S.D. were determined for each treatment time and significance determined by Student's "t" test.

Toxicity Assays

Chemical toxicity was determined by seeding 8 wells with 500 cells per well as described for transformation assays. Cells were treated 90 min., washed and refed with CM + 20% FBS. Cultures were incubated 9-10 days, fixed, stained with Giemsa and clones containing at least 50 cells were counted. Toxicity was determined by dividing the average number of clones in treated wells by the average number of clones in control wells.

Chemicals

NA, PANA, PBNA, MANA and DS were dissolved in spectral grade acetone at 1.0 mg/ml. Prior to use, dilutions were made in CM to experimental concentrations. SDMH was dissolved in 0.1 N HCl at a concentration of 10 mg/ml and diluted in CM to experimental concentrations.

Both petroleum or shale oil-derived fuels, JP5, diesel fuel, marine, RJ5 and JP10, were dispersed in serum-free CM with shaking. Dilutions tested were from 5,000 to 0.5 ppm. Eight wells, seeded with 500 cells/well, were treated for 90 min., washed and refed with CM supplemented with 20% FBS. Cultures were incubated 9-10 days, fixed in 10% buffered formalin, stained with Giemsa, and clones containing at least 50 cells were counted. Toxicity was determined by dividing the average number of clones in treated wells by the average number of clones in control wells. LD_{50} values were determined by plotting percent survival (ordinate) against concentration (abcissa, log scale) on 3-cycle semi-logarithmic graph paper. LD_{50}'s were then determined by inspection.

RESULTS

Petroleum-derived (PD) or shale oil-derived (SD) JP5 or diesel fuel, marine (DFM) cytotoxic analyses are shown in Table 1. The results show no significant difference in toxicity values between SD or PD fuels. LD_{50}'s for SD, JP5 and PD-JP5 were 102 ppm and 100 ppm, respectively, SD-DFM and

and PD-DFM were 85 ppm and 87 ppm, while LD_{50}'s for JP10 and RJ5 were 91 ppm and 19 ppm, respectively.

TABLE 1

LD_{50} CYTOTOXICITY OF SHALE OIL AND PETROLEUM-DERIVED FUELS IN HSF CELLS.

Fuel	Derived From	LD_{50} (ppm)
JP5	Shale	102
JP5	Petroleum	100
DFM	Shale	85
DFM	Petroleum	87
RJ5	Petrolem	19
RJ10	Petroleum	91

PANA treatment resulted in a dose-related suppression of transformation (Fig. 1A). Cells treated with 10 µg/ml resulted in significant inhibition of transformation at all time periods tested. Results with 20 µg/ml were similar, although treatment at 6 hrs. p.i. approached control values. Cells treated with 0.1 µg/ml showed no difference in transformation frequencies from untreated controls.

Treatment with 10 µg/ml PBNA resulted in suppression at all time periods tested (Fig. 1B). Inhibition of transformation ranged from values of 40% to 65% inhibition. However, cells exposed to 0.01 ug/ml before ST-FeSV infection resulted in enhanced focus formation at -2 hrs., whereas significant inhibition was observed when virus-infected cells were treated 6 hrs. p.i.

Treatment with 50 or 5 ppm PD-JP5 at all time periods tested resulted in no differences in transformation frequencies from control values (Fig. 2). ST-FeSV transformation frequencies were significantly reduced when cells were treated with either 50 or 5 ppm SD-JP5 24 hrs. prior to virus infection, whereas transformation frequencies were similar to control values at the other time periods tested.

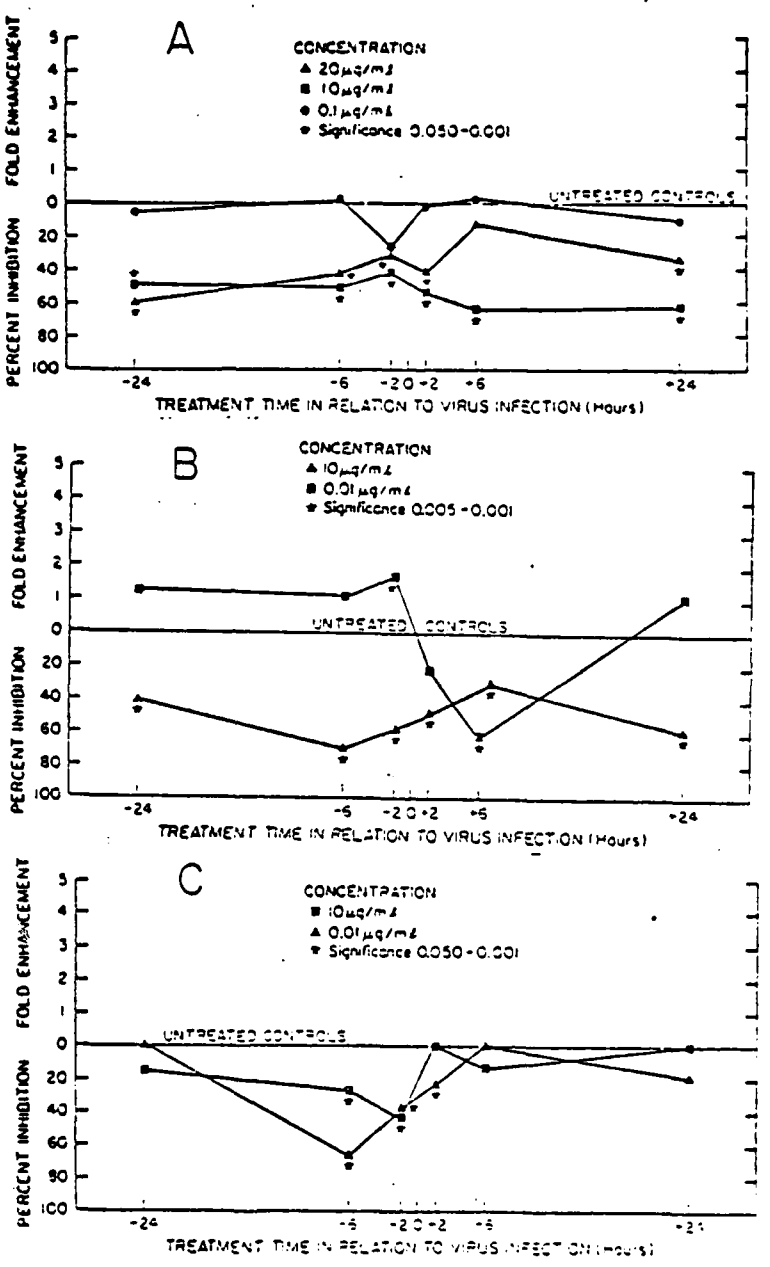


FIGURE 2. HSF cells were plated in 16 mm diameter wells with 2.0 ml CM and incubated overnight. Cells were treated as described in Materials and Methods. A-PANA, B-PBNA and C-NA.

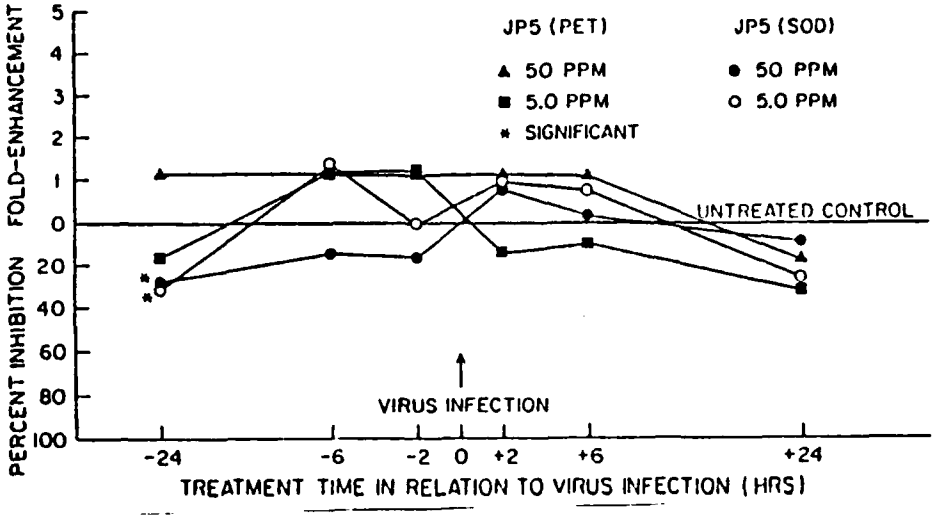


FIGURE 2. Petroleum and shale oil-derived JP5 effects on ST-FeSV transformation. Assay described in Materials and Methods.

The biological specificity of the assay is shown by the data in Tables 2 and 3. Ten ug/ml SDMH or 10 µg/ml SDMH mixed with 0.01 µg DS were added to HSF 2 hrs. p.i. (Table 2). As shown in Table 2, SDMH reduced the transformation frequency by 44% (p=0.001), whereas co-treatment with SDMH + DS resulted in a non-significant 22% difference in the transformation frequency (p=N.S.). One ppm MAMA or 1 ppm

TABLE 2

ABROGATION OF THE INHIBITORY EFFECT OF SDMH ON ST-FeSV TRANSFORMATION BY DS.

Chemical Treatment	FFU/ml X 10^{-1}	% Reduction in FFU	$(p)^2$
SDMH	1.41	44	0.001
SDMH + DS	1.99	27	N.S.
ST-FeSV	2.51	-	-

1. Twelve wells infected with 30 FFU for each chemical treatment. SDMH (10 µg/ml) and/or DS (0.01 ug/ml) added 2 hr. p.i. as described in Materials and Methods.
2. (p) = Student's "t" test.

MAMA mixed with 0.01 µg/ml DS reduced the transformation frequencies by 41% (p=0.050) and 37% (p=0.010), respectively (Table 3).

TABLE 3

FAILURE OF DS TO ABROGATE THE INHIBITORY EFFECT OF MAMA

Chemical Treatment	FFU/ml X 10^{-1}	% Reduction in FFU	(p)[2]
MAMA	1.10	41	0.050
MAMA + DS	1.19	37	0.010
ST-FeSV	1.86	-	-

1. Cells treated as described in Table 2. MAMA-1 ppm, DS-0.01 µg/ml.
2. (p) = Student's "t" test.

DISCUSSION

Cytotoxicity studies of water soluble fractions of SD or PD-derived JP5 fuels and DFM showed no differences in cellular toxicity. Effect on ST-FeSV transformation by SD or PD-derived JP5 were negative. In vivo animal studies reported by Rowland et al. (5) with 4 fractions from shale oil extracted in benzene reported only shale oil coke extract was carcinogenic and contained high concentrations of BAP. Major water soluble fractions of JP5 fuel contain no BAP, but do contain several aromatic components, none of which have been assayed in this system (6).

Methylazoxymethanol (MAM) is believed to be the proximate carcinogen of SDMH and the ultimate carcinogen, a methyldiazonium ion formed when the MAM undergoes oxidative metabolism (7). Thus, the biological specificity of this assay is suggested by the data shown in Tables 2 and 3. The inhibitory effect of SDMH on virus transformation was abrogated by co-treatment with DS (Table 2), whereas the inhibitory effect of MAMA on virus transformation was not abrogated by DS (Table 3). Because DS inhibits biotransformation of SDMH at the azomethane to azoxymethane step, co-treatment of DS and MAMA should not abrogate inhibition of virus transformation (7).

Contrasting results have been reported on the interaction of chemical carcinogens and retroviruses. For example, in

example, in vivo studies showed either an inhibitory (8,9), co-carcinogenic (10,11), or no effect (12,13) on transformation, depending upon the virus or chemical used in the experiments. In vitro studies with rat or mouse cells and viruses showed synergism (14-16).

This HSF and ST-FeSV system show a time-dependent and, in most cases, a dose-dependent relationship in which diverse classes of chemicals (Table 4) inhibited ST-FeSV transformation when virus-infected cells were treated with non-toxic doses of chemicals at 2-6 hrs. p.i. Since the inhibitory effect was abrogated when cells were exposed >24 hrs. p.i., the temporal relationship between infection and treatment suggest chemical interference with either FeSV proviral synthesis or integration into host cell DNA.

TABLE 4

CHEMICALS TESTED IN VIRUS INHIBITION ASSAY.

Chemical Group	Carcinogenic Activity	Inhibition of Virus Transformation
I. Aromatic Amines		
A. Naphthyl Amines		
1. Two (2)	(+)[1]	+
2. Phenyl-alpha	(+)	+
3. Phenyl-beta	(+)	+
B. N-Acetoxy 2 fluor-enylacetamide	(+)	+
II. Polycyclic Hydrocarbons		
1. Benzo(a)pyrene	(+)	+
2. Pyrene	(-)	-
III. Hydrazines		
1. Hydrazine	(+)	+
2. Mono-methyl hydrazine	(+)	+
3. 1,1 dimethyl hydrazine	(+)	+
4. 1,2 dimethyl hydrazine	(+)	+
IV. Other		
1. Aflatoxin B_1	(+)	+
2. Amosite Asbestos	(+)	+
3. JP5 (SD)	(-)	-
4. JP5 (PD)	(-)	-
5. RJ5 (TH dimer)	(-)	-
6. Diesel fuel, Marine	(-)	-
7. Acetone	(-)	-
8. Methyl Azoxymethanol-acetate	(+)	+
9. Formalin	(+)	+
10. Triton X-100	(?)	-

1. (+) considered a carcinogen, (-) non-carcinogen, from
(1) Advances in Modern Environmental Toxicology, Vol. I. Eds, N. Mishra, V. Dunkel, M. Mehlman. Senate Press, N.J. 1980.
(2) "Origins of Human Cancer", by I.J. Selikoff, Eds, H. Hiatt, J.D. Watson, J.A. Winsten. Cold Spring Harbor, N.Y. 1977.
(3) Unpublished data from Toxicology Branch, AMRL, WPAFB.

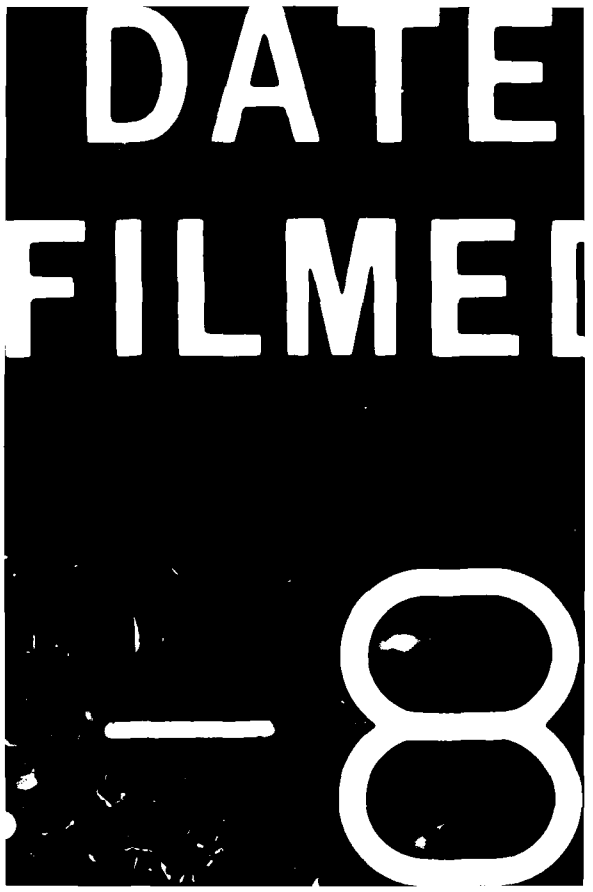
ACKNOWLEDGEMENTS

Supported by Air Force Office of Scientific Research Contract F49620-80-C-0087. We thank Wendy G. Sowder, Stephen A. Smith and Janice Wagner for excellent technical assistance.

REFERENCES

1. Blakeslee, J.R., and Milo, G.E. (1978): Feline sarcoma virus in vitro infection of human cells. Influence of chemical carcinogens on focus formation. Chem-Biol. Interactions 23, 1-11.
2. Blakeslee, J.R., Elliot, A.M., and Turner, D.T. (1980): Induction of retrovirus non-producer human cells to producer human cells by dexamethasone. In: Advances in Comparative Leukemia Research, Eds. D.S. Yohn, B. Lapin and J. Blakeslee, pp. 87-89, Elsevier North Holland, New York.
3. Blakeslee, J.R., Elliot, A.M., and Carter, L.J. (1982): Anti-oncogenic activity of diverse chemical carcinogens on retrovirus-induced transformation of human skin fibroblasts. Annals of the N.Y. Acad. of Sci. (in press).
4. Schaller, J.P., Milo, G.E., Blakeslee, J.R., Olsen, R.G., and Yohn, D.S. (1976): Influence of glucocorticoid estrogen and androgen hormones on transformation of human cells in vitro by feline sarcoma virus. Cancer Res. 36, 1980.
5. Rowland, J., Shubik, P., Wallcave, L., and Sellakumar. (1980): Carcinogenic bioassay of oil shale: Long-term percutaneous application in mice and intratracheal instillation in hamsters. Toxic and Appl. Pharm. 55, 522-534.
6. Smith, J., and Harper, J. (1981): Behavior of hydrocarbon fuels in aquatic systems. In: AF AMRL-TR-81-149, Proceed. of the XII Conf. on Env. Toxic. pp. 336-353.
7. Fiala, E.S. (1977): Investigations into the metabolism and mode of action of the colon carcinogens 1,2 dimethylhydrazine and azoxymethane. Cancer 40, 2436-2445.
8. Whitmire, C.E., and Huebner, R.J. (1972): Inhibition of chemical carcinogenesis by viral vaccines. Science 177, 60.
9. Laro, L.W., and Precerutti, A. (1963): Inhibition by urethan (ethyl carbamate) of virus induction of leukemia in C_3H mice. Nature 200, 692.

10. Engle, C.G., and Groupe, V. (1969): Effect of chemical carcinogenesis on virus-induced Rous sarcoma. Cancer Res. 29, 1345.
11. Vesselinovitch, S.D., Simmons, E.L., Miharlovich, N., Lombard, L.S., and Rao, K.V. (1972): Additive leukemogenicity of urethan and X-irradiation in infant and young adult mice. Cancer Res. 32, 222.
12. Nowinski, R.C., and Miller, E.C. (1976): Endogenous oncornaviruses in chemically induced transformation. II. Effects of virus production in vivo. J. Natl. Cancer Inst. 57, 1347.
13. Gross, L., Roswit, B., Mada, E.R., Dreyfuss, Y., and Moore, L.A. (1971): Studies on radiation-induced leukemia in mice. Cancer Res. 19, 316.
14. Rhim, J.S., Vass, W., Cho, H.Y., and Huebner, R.J. (1971): Malignant transformation induced by 7,12 dimethylbenz(a)-anthracene in rat embryo cells infected with Rauscher virus. Int. J. Cancer 7, 65.
15. Price, P.J., Suk, W.A., Peters, R.L., Gilden, R.V., and Huebner, R.J. (1977): Chemical transformation of rat cells infected with xenotropic type C-RNA virus and its suppression by virus-specific antiserum. Proc. Natl. Acad. Sci. USA 74, 579.
16. Price, P.J., Suk, W.A., and Freeman, A.E. (1972): Type C RNA tumor viruses as determinants of chemical carcinogenesis: Effects of sequence of treatment. Science 177, 1003.


DATE
FILME
8