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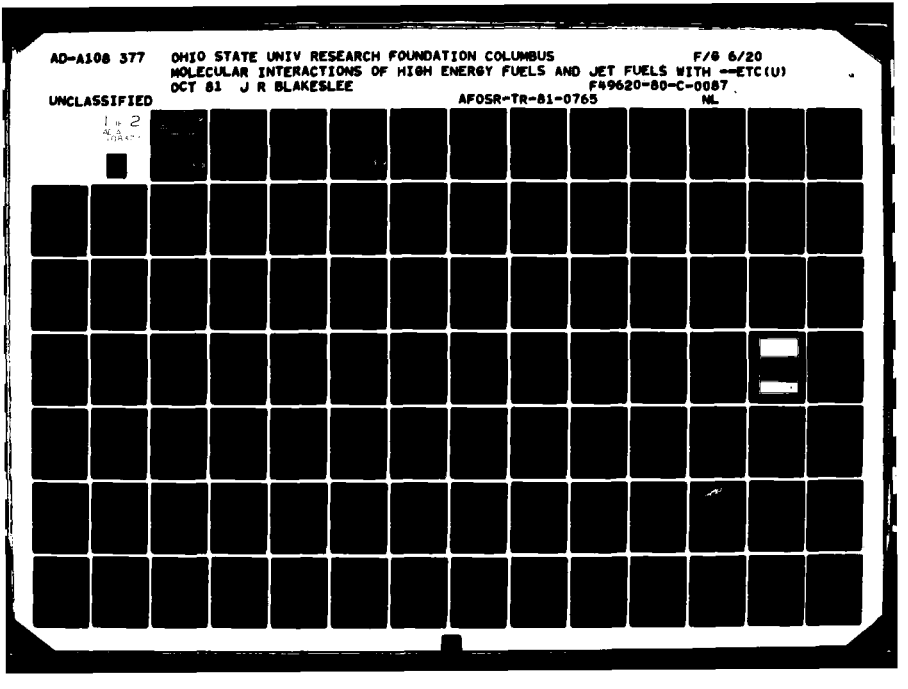

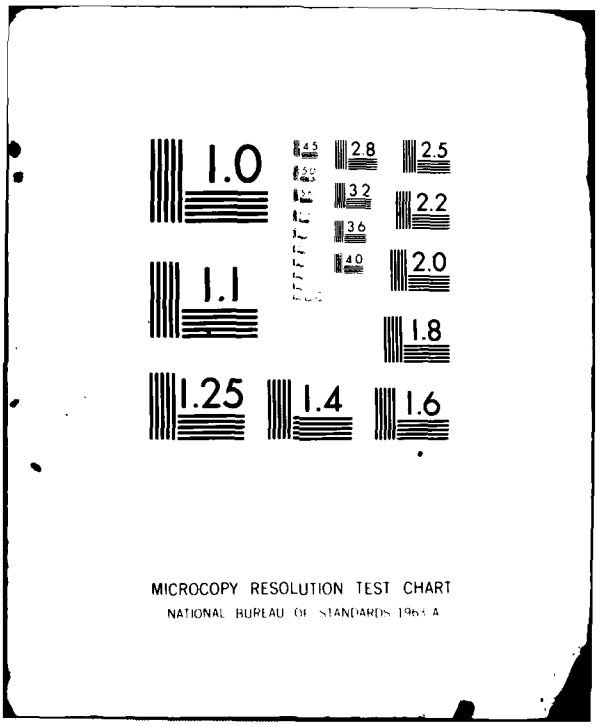
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MOLECULAR INTERACTIONS OF HIGH ENERGY FUELS AND JET FUELS WITH ONCOGENIC VIRUSES AND ENDOGENOUS VIRUSES

James R. Blakeslee
Department of Veterinary Pathobiology

For the Period
July 31, 1980 - September 15, 1981

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

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October, 1981

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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.

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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. Isolation and purification of an endogerous feline virus RD114 structural protein p 28 is described. P28 is expressed in all feline tissues and cells. The cat cells will be treated with chemical carcinogens and non-carcinogens and the effect on P28 expression will be determined by competitive radioimmune assays. Increased or decreased P28 expression will be correlated with carcinogenicity of test chemical.

PROGRESS REPORT

TO

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

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

Inclusive Date of Report:
July 31, 1980 to September 15, 1981

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I. Research Objectives

A. 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 in their worldwide operations. The development of such assays to evaluate and determine carcinogenic potential of a chemical would greatly reduce the time and money now required to determine the biological effects in in vivo animal systems. These studies involved two separate systems, both involving in vitro assays. The one system has been used to investigate the effect diverse chemicals have on a known transforming oncogenic virus in human cells. To date, the results of these experiments show chemical carcinogens inhibit oncornavirus transformation of human cells when virus-infected cells are treated between 2 and 6 hours post-infection, while non-carcinogenic chemicals do not. The second approach, in the development stage, has been to investigate effects on gene expression of a known viral gene product as a function of carcinogenic potential. The results of both of these studies would lead to the establishment of qualitive predictable parameters as to potential carcinogenic action and lead to the understanding of the complex interactions between normal cellular processes and environmental carcinogens - the implication being prevention of the carcinogenic process.

B. Specific Objectives

The specific aims of this contract are to:

1. Continue evaluation of virus-transforming assay system as a screen for chemical carcinogens. More carcinogenic and noncarcinogenic logs will be assayed in order to validate the system. Hydrazine, symmetrical dimethyl hydrazine, monomethyl hydrazine, unsymmetrical dimethyl hydrazine, and radiolabeled compounds, that is of the hydrazines, from Dr. Donald Witiak's laboratory, in addition to jet fuels, will be evaluated.

2. Determine whether hydrazine, symmetrical dimethyl hydrazine, unsymmetrical dimethyl hydrazine, monomethyl hydrazine, JP 10, RJ 5, (petroleum and shale oil derived) or other fuels or hydrazine metabolites from Dr. D. Witiak's laboratory, alter the expression of the endogenous feline virus RD114, translational protein P28, by competitive radioimmune assay and/or the transcriptional viral RNA by C-DNA hybridization as a predictive test for carcinogenicity of these compounds.
3. Determine molecular interactions of hydrazine and the radioactive hydrazines described in No. 2 with FeSV proviral synthesis or integration to radiolabeled molecular and metabolic probes.
4. Determine whether molecular events described in No. 3 are altered when synchronized cell populations are used as opposed to unsynchronized populations.

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 storage in order that these passage levels can be used. The cell line RD114/RD, a human rhabdomyosarcoma cell line shedding the RD114 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. Chemicals tested.

During this past year the following chemicals were tested and in the virus transformation assay system:

1. Hydrazine (Hż), 1,2 symmetrical dimethyl hydrazine (SDMH), 1,1, unsymmetrical dimethyl hydrazine (UDMH), monomethyl hydrazine (MMH), JP 5 jet fuel (petroleum derived and shale oil derived), RJ 5 fuel and diesel fuel, marine. Other chemicals assayed were Triton X-100, formalin, spectrophotometric grade acetone and methyl azoxy methanol acetate. Other chemicals used in these studies were ^{14}C double-labeled SDMH prepared by Dr. Fred Cazer in Dr. Donald Witiak's laboratory and used to determine whether disulfiram inhibited DNA methylation by SDMH. Also participating in this study was Dr. William Gower, Department of Medicinal Chemistry. Chemicals were diluted in complete Minimum Essential Medium with Earles salts supplemented with 50 µg/ml of gentamicin sulfate, and with 10% heat-inactivated fetal bovine serum prior to use in any of the in vitro experiments.

C. Results of experimentation.

1. Isolation and purification of the RD114/P28 structural protein.

The initial stages of this study have concentrated on the production of RD114 virus and the subsequent isolation of the structural protein, P28. The RD114 virus is continuously released from the human rhabdomyosarcoma cell line, RD114/RD. This cell line was obtained from the biological carcinogenesis program of Frederick Cancer Research Center. After discussion with Sandra West of the Frederick Cancer Research Center, a procedure was developed and is now routinely used to produce and isolate the RD114 virus. The RD114/RD cells are seeded in 175 cm^2 Falcon Tissue Culture flasks at a concentration of $1x10^7$ cells per flask. Once the cells have reached confluency they begin to shed virus. At this time, the medium is removed and the cells are fed at 48 hour intervals for 20 days. The harvested culture media is stored at 4^oC. The culture media is then concentrated on a Millipore Pellicon Cassette System from its original volume of approximately 10 liters to 200 ml,

followed by ultracentrifugation (100,000 x g, 90 minutes) to pellet virus. The virus is then resuspended and purified by centrifugation on a continuous sucrose gradient.

The second stage of this study has dealt with the isolation of the structural protein P28. A crude extract of the RD114 virus was first produced according to the procedure described by Mathes et al., 1979. The virus was freeze-thawed twice, followed by ultracentrifugation (100,000 x g, 90 minutes). The resulting pellet was resuspended in TKE-D-Tx buffer (0.05M Tris, pH 7.2, 0.6M KCl, 0.01M EDTA, 0.01M Dithiothreitol, 1% Triton X-100), incubated at 37C for 60 minutes and then ultracentrifuged (100,000 x g, 90 minutes). The resulting supernate was extracted with ether, to remove the Triton X-100 and then bubbled with N_2 to remove the ether. This was followed by ultracentrifugation (100,000 x g, 90 minutes) and the resulting supernate was termed "Fraction C". Fraction C contained approximately 2.0 mg of protein and produced 8 distinct bands on SDS gel elctrophoresis. The most intense band had a molecular weight of approximately 25,000 to 30,000 daltons, and is believed to represent the RD114 structural protein P28. The other seven proteins had molecular weights of 10,000, 13,500, 16,000, 31,000, 43,000, 55,000 and 73,000, respectively.

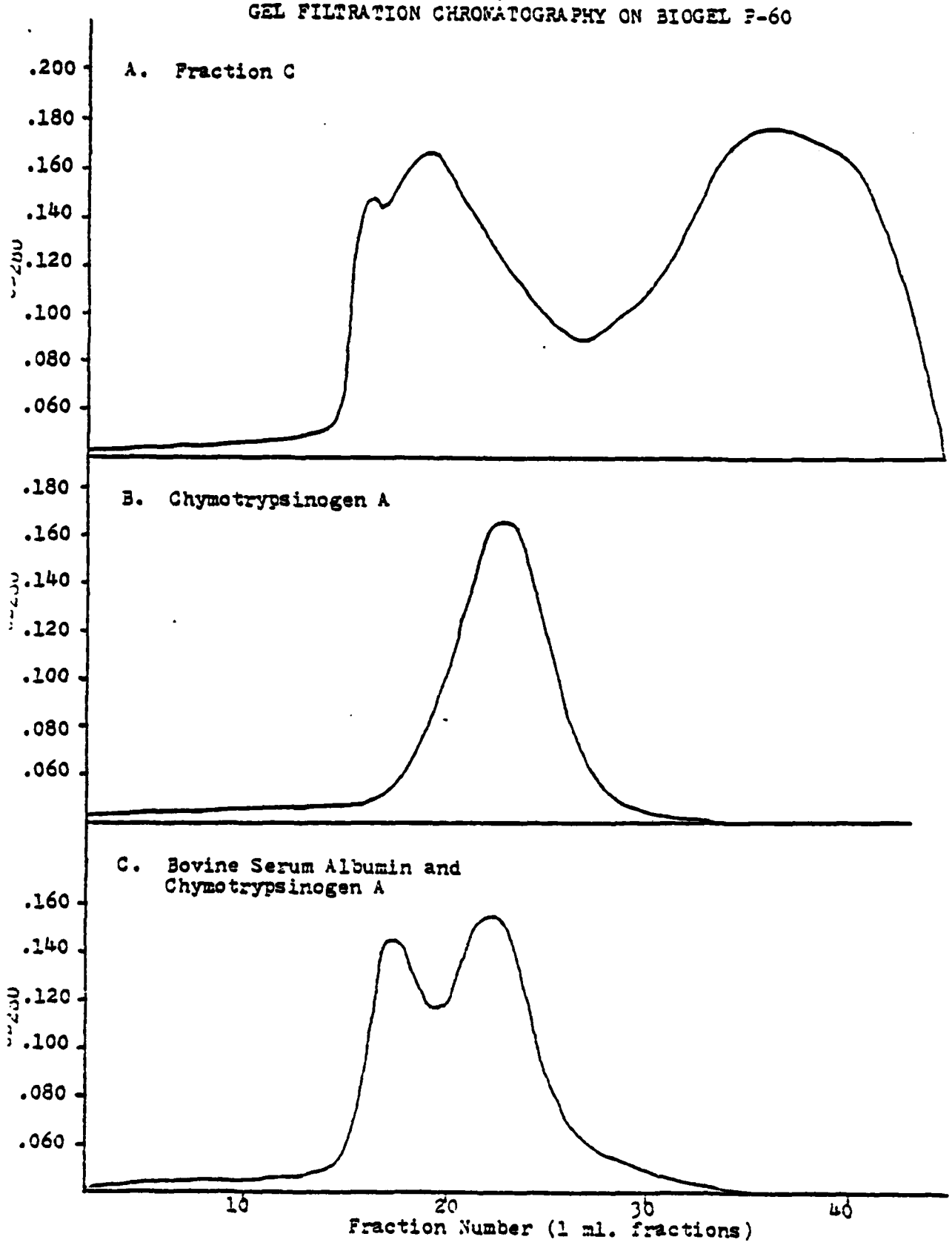
In an attempt to isolate P28 from the other proteins present in Fraction C, we initially followed the procedure of Mathes et al., 1979. Mathes successfully isolated the Feline Leukemia Virus P27 protein by gel filtration chromatography on a Sephacryl S-200 column (2.6 x 100 cm). When Fraction C isolated from the RD114 virus was chromatographed on a comparable column, protein was not detected in the elution volume by UV absorption. This inability to detect the sample in the elution volume could be corrected by using a smaller column. A reduction in column size would reduce the sample dilution and possibly permit detection of protein by UV absorption. Alternatively, Fraction C could be radiolabeled with ^{125}I prior to gel

filtration chromatography. Protein elution could then be monitored by measuring radioactivity in the elution volume. Both a reduction in column size and radiolabeling of Fraction C were tested in attempts to isolate P28 from Fraction C. Preliminary results indicate a reduction in column size permit detection of protein in elution volume by UV absorption. The radiolabeling of Fraction C for the purpose of isolating P28 is not necessary.

Figure A is the elution profile obtained when Fraction C isolated from RD114 virus was chromatographed on a Biogel P-60 column (1.5 x 30 cm). Standards of known molecular weights were also chromatographed on the Biogel P-60 column. Figure B is the elution profile obtained when chymotrypsinogen A (molecular weight 25,000 daltons) was chromatographed on the Biogel P-60 column described above. When a mixture of chymotrypsinogen A and bovine serum albumin (molecular weight 68,000 daltons) was chromatographed, the elution profile (Figure C) contained two peaks. The first peak (fractions 15 to 20) eluted in the void volume and contained the bovine serum albumin. The second peak (fractions 20 to 25) contained chymotrypsinogen A. Based on the elution pattern of chymotrypsinogen A on the Biogel P-60 column, the P28 protein isolated from the RD114 virus should elute in fractions 20 to 25. When Fraction C isolated from RD114 was chromatographed on Biogel P-60 (Figure A) the resulting fraction 20 produced a single band in SDS gel electrophoresis. The protein in this band had a molecular weight of approximately 25,000 to 30,000 daltons which suggested that this band represented the structural protein P28.

P28 had been isolated in fraction 20; however, the separation of protein which was obtained on the Biogel P-60 column was not totally satisfactory. When Fraction C isolated from the RD114 virus was chromatographed on Biogel P-60, the shape of the resulting elution profile (Figure A) suggested the presence of contaminating proteins

GEL FILTRATION CHROMATOGRAPHY ON BIOGEL P-60



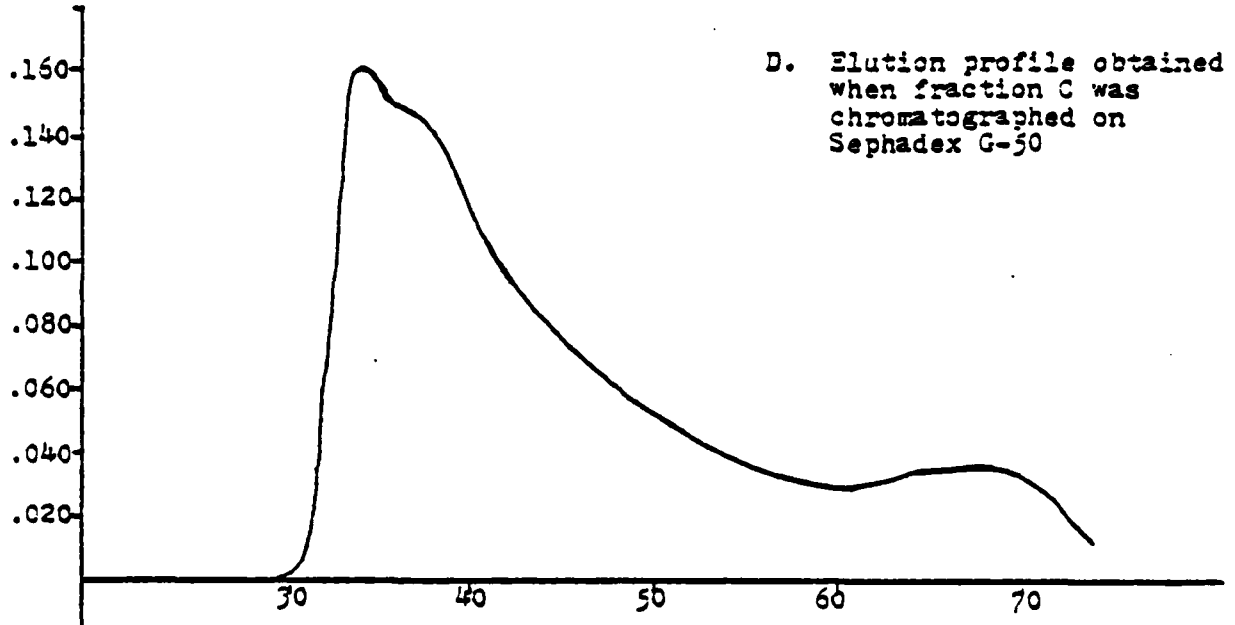
in the P28 peak (fractions 15 to 25). This suspicion was confirmed when SDS gel electrophoresis was performed on all the fractions in this peak. The P28 protein was detectable in all eleven fractions with the highest level found in fractions 19, 20 and 21. Fractions 21 through 25 contained two other proteins with molecular weights of approximately 70,000 and 55,000 daltons.

A new isolation procedure was developed in an attempt to remove these contaminating proteins and still obtain a maximum yield of P28. The crude Fraction C was produced from RD114 virus as described above, and 2.5 ml aliquots of Fraction C were chromatographed on a Sephadex G-50 column (1.5 x 45 cm). This column should exclude the P28 protein and successfully separate P28 from contaminating proteins of lower molecular weight. Figure D represents the elution profile obtained when Fraction C was chromatographed on Sephadex G-50. SDS gel electrophoresis of selected fractions demonstrated the presence of P28 in the first eluted peak. This peak also contained three other proteins with molecular weights greater than 30,000 daltons; however, the contaminating proteins with molecular weights lower than 25,000 daltons were not detected in the first eluted peak.

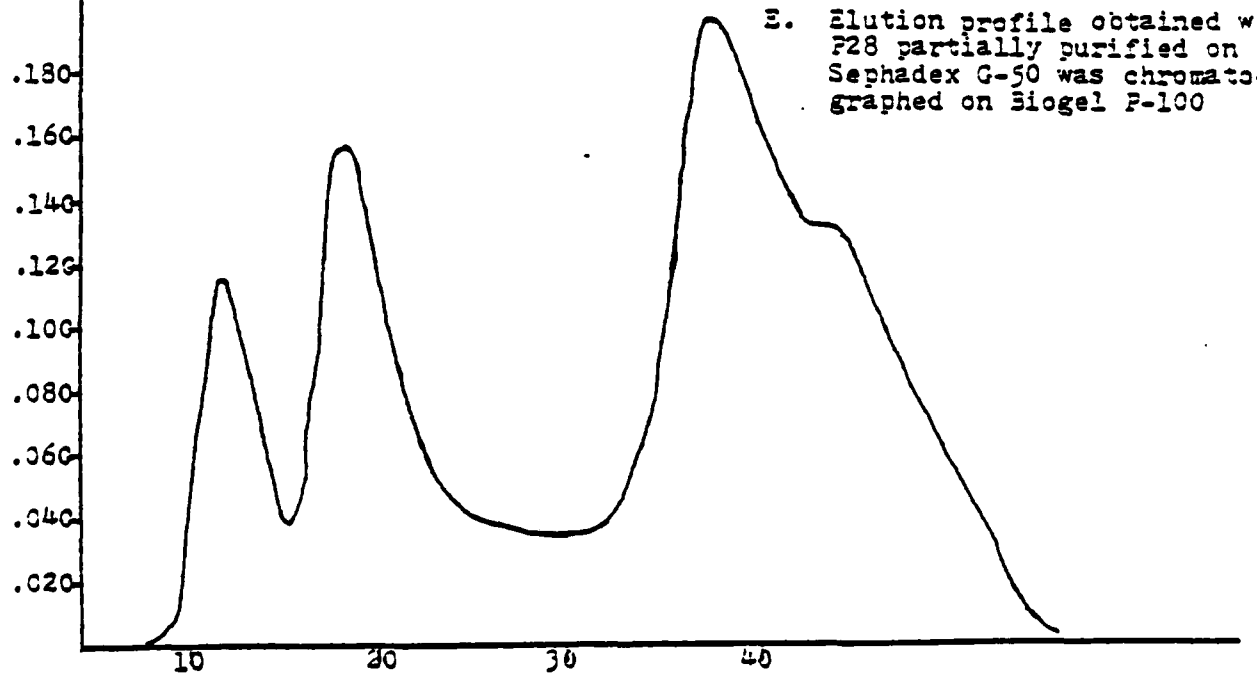
An attempt was then made to separate the P28 protein from the three contaminating proteins with molecular weights greater than 30,000 daltons. Fractions 32 through 39 from the first eluted peak were pooled and concentrated. The concentrated fractions were then chromatographed on a Biogel P-100 column (1.5 x 30 cm). The resulting elution profile is shown in Figure E. SDS gel electrophoresis of selected fractions demonstrated the higher weight protein contaminates had eluted in the first peak (fractions 10 to 15). Only the P28 protein was detected in the second eluted peak. Fractions 17 through 20 from the second eluted peak were pooled and chromatographed on a Sephadex G-25 (eluting buffer of 0.1M ammonium bicarbonate)

Mathes, L.E., Olsen, R.G., Hebebrand, L.C., Hoover, E.A., Schaller, J.P., Adams, P.W., Nichols, W.S. 1979. Immunosuppressive Properties of a Virion Polypeptide, a 15,000 Dalton Protein, from Feline Leukemia Virus. Cancer Research 39:950-955.

GEL FILTRATION CHROMATOGRAPHY



D. Elution profile obtained when fraction C was chromatographed on Sephadex G-50



E. Elution profile obtained when P28 partially purified on Sephadex G-50 was chromatographed on Biogel P-100

Fraction Number (1 ml. fractions)

to remove the salts present in the P28 preparation. The purified P28 protein was then lyophilized and stored at -70°C.

C. Results of experimentation (continued)

2. Methods and results of research on the enhancement of transformation and genome integration into Detroit 550 Cellular DNA by feline sarcoma virus in the presence of carcinogens.

a. Culture of Detroit 550 (D550) Human Foreskin Cells (American Type Culture Collection).

Cells are grown in Corning 490 cm^2 tissue culture roller flasks on a roller apparatus in 100 ml per flask of the following complete medium:

Component	% of Total Volume
Minimum Essential Medium with Earle's salts (Gibco) (MEM-E with 0.22% NaBicarbonate)	86.9%
Fetal Bovine Serum (FBS)	10.0%
Non-Essential Amino Acids (M.A. Bioproducts)	1.0%
Sodium Pyruvate (M.A. Bioproducts)	1.0%
L. Glutamine (Gibco)	1.0%
Gentocin (Antibiotic)	0.1%

Cells are seeded at approximately $6x10^6$ cells per roller flask and grown to confluency. The cells from each confluent flask are passaged (split) into 2 to 3 flasks until adequate numbers have been grown for co-carcinogenesis transformation studies. (Approximately one week is required for the cells to reach confluency.)

b. Procedure for co-carcinogenesis transformation of Detroit 550 cells.

Forty tissue culture roller flasks at approximately 80% confluence ($10x10^6$ cells per flask) are used for each transformation experiment to provide sufficient cellular DNA for subsequent hybridization experiments.

Co-carcinogenesis transformation is being done using Feline Leukemia Virus (Snyder-Theilen strain) (FeLV-ST) and each of the following compounds at the indicated concentrations and times relative to FeLV-ST infection:

Compound	Concentration Administered	Time Administered Relative to FeLV-ST Infection
Hydrazine	60 ppm	- 2 hours*
		+ 2 hours*
Methyl Hydrazine	100 ppm	- 2 hours*
		+ 2 hours*
Benzo(a)pyrene	10 µg/ml	- 2 hours*
		+ 2 hours*
Pyrene	10 µg/ml	- 2 hours
		+ 2 hours
Unsymmetrical Dimethyl Hydrazine	100 µg/ml	- 6 hours
		+ 6 hours
Symmetrical Dimethyl Hydrazine	100 µg/ml	- 6 hours
		+ 6 hours

Control Experiments:

1) D 550 cells infected with FeLV-ST only (no carcinogenic compound added) - D 550 cells are infected at 80% confluency and grown 6 days.*
2) D 550 cells are grown to normal confluency with no FeLV-ST and no compound added.*

*These co-carcinogenesis transformation and control experiments have been completed and harvested cells are stored frozen at -80°C for future DNA extraction. The remaining co-carcinogenesis transformation studies are in progress.

Step by step procedure involved in the co-carcinogenesis transformation of Detroit 550 cells.

Have ready 40 roller flasks of D 550 cells at 80% confluency of growth (about $10x10^6$ cells per flask).

1) Procedure for administration of compound 2 hours prior to FeLV-ST infection:

   a) Aspirate medium from roller flasks.

   b) Add 20 ml of compound (co-carcinogen) at the desired concentration in complete MEM-E/Na medium containing 10% fetal bovine serum (FBS) to each roller flask and incubate at 37°C for 1.5 hours.

   c) Aspirate compound (co-carcinogen) off.

   d) Add DEAE-Dextran at 40 µg/ml prepared in incomplete MEM-E/Na medium (no FBS) using 5 ml per culture flask. (DEAE-Dextran enhances virus absorption and penetration of the cells). Incubate at 37°C for 20 minutes.

   e) Aspirate off DEAE-Dextran.

   f) Infect D 550 cells with FeLV-ST at approximately $1.22x10^3$ focus-forming units/ml (FFU/ml) in MEM-E/Na medium containing 5% FBS using 5 ml per culture flask. Incubate at 37°C for 2 hours.

   g) Aspirate off virus.

   h) Add 50 ml of complete MEM-E/Na medium (10% FBS) to each culture flask. Allow cells to grow an additional 6 days at 37°C.

   i) Harvest cells by trypsinization, pellet by low speed centrifugation (1,000 rpm for 10 minutes), and store frozen at -80°C.

c. Procedure for extracting cellular DNA from D 500 cells used in co-carcinogenesis transformation experiments.

These methods are modifications of those reported in the following references:

1) Britten, R.M., Graham, D.E. and Neufeld, B.R. 1974. Analysis of Repeating DNA Sequences by Reassociation. In Methods in Enzymology. Vol. 29, Part E: Capt. 29:375-378.

2) Fujinaga, K., Rankin, A., Yamazaki, H., Sekikawa, K., Bragdon, J. and Green, M. 1973. RD114 Virus: Analysis of Viral Gene Sequences in Feline and Human Cells by DNA-DNA Reassociation Kinetics and RNA-DNA hybridization. Virology 56:484-495.

Nuclease free glassware (siliconized also) is used throughout. Unless otherwise indicated all extraction steps are done at room temperature:

1) The cellular pellet is suspended in 1X TNE buffer (0.1 N Tris, 0.1M NaCl, 0.0001M EDTA) pH 9.0.

2) The cellular suspension is made 0.5% with SDS (sodium dodecyl sulphate) by adding one-tenth volume of 5.0% SDS prepared in 1X TNE, pH 9.0.

3) An equal volume of Pronase (nuclease free and self-digested at 37°C for 2 hours; CalBiochem) or Proteinase K (self-digested at 60°C for 20 min; Boehringer Mannheim) at 500 µg/ml in 1X TNE, pH 9.0 is added to the suspension. Final enzyme concentration in the suspension is 250 µg/ml. The mixture is then incubated at 37°C for 2 hours.

4) Three volumes of TNE, pH 9.0 saturated-phenol: chloroform: isoamyl alcohol (25:24:1) are added to the suspension. This mixture is allowed to stir slowly at room temperature for 30 minutes.

5) Centrifuge at 2,000 rpm for 10 minutes to separate the upper aqueous and more dense lower organic phases. Carefully remove upper aqueous layer with a pasteur pipet.

6) Re-extract the aqueous phase as in step 4 and centrifuge as in step 5 two more time to remove all protein (3 phenol extractions in total).

7) Two volumes of cold absolute ethanol are added to the aqueous phase.

8) The aqueous phase plus ethanol is made 0.2M with sodium acetate by adding one-tenth volume of 2M Na acetate.

9) The mixture is stored at -20°C for at least 24 hours to allow for precipitation of the DNA. DNA is then eitehr stored in the ethanol at -12°C or as in step 10.

10) DNA may be spooled out and frozen in a small volume of 0.1 X SSC (SSC = 0.15M NaCl, 0.015M Na citrate), or in 1X TE buffer (0.010M Tris, 0.005M EDTA, pH 7.4) at -80°C.

11) DNA concentration is determined by optical density at 260 nm (1 OD_{260} = approximately 50 µg DNA) or by the diphenylamine reaction using polymerized calf thymus DNA (Sigma) as a standard.

12) DNA preparations at this point should be tested for the presence of RNA, contaminating proteins, and degradation. Preliminary testing can be done by melting the DNA optically.

A pure DNA preparation should demonstrate:

a) hyperchromicity of 25-26%,

b) the T_m (melting temperature) expected for the GC content of the DNA (diploid mammalian DNA), and

c) presuming that there are no AT-rich satellites or components, no hyperchromic shift should appear until most of the preparation starts to melt.

RNA contamination can be eliminated by treatment with RNase A (50 to 100 µg/ml) for 2 hours at 37°C. Further treatment with Pronase or Proteinase K can be done to remove contaminating proteins.

d. <u>Culture of FL-74 cells which continually produce and shed Feline Leukemia Virus-Kawakami-Theilen Strain (FeLV-KT)</u>:

Cells are grown in suspension in 0.5 gallon disposable glass roller bottles on a roller apparatus at 37°C in the following complete medium:

Component	% of Total Volume
RPMI 1640 (Gibco) containing 0.2% Na Bicarbonate	82.9%
Newborn Calf Serum	15.0%
L-Glutamine (Gibco)	1.0%
Penicillin (at 200,000 units/ml)	1.0%
Gentocin (Antibiotic)	0.1%

Cells are seeded weekly (7 day intervals) at 1.2 to 1.4 x 10^6 cells/ml in 200 ml of the RPMI 1640 complete medium per roller bottle. On day 5 the cells are fed an additional 200 ml per roller bottle of the RPMI 1640 complete medium.

The medium-containing virus, which is continually shed by the cells, is harvested on day 8 and cells are removed by centrifugation at 1,500 rpm for 15 minutes. The medium may be stored at 4°C for short periods of time (up to one week) prior to virus purification.

e. Purification procedure for Feline Leukemia Virus - KT strain

These methods are based upon those presented in the following references:

1) Thomason, A.R., Friderici, K.H., Velicer, L.F. and Rottman, F. 1978. Presence of 5' - Terminal Cap Structures in Virus-Specific RNA From Feline Leukemia Virus-Infected Cells. Journal of Virology Vol. 26(2):226-235.

2) Thomason, A.R., Brian, D.A., Velicer, L.F. and Rottman, F.M. 1976. Methylation of High-Molecular-Weight Subunit RNA of Feline Leukemia Virus. Journal of Virology Vol. 20(1):123-132.

3. Brian, D.A., Thomason, A.R., Rottman, F.M. and Velicer, L.F. 1975. Properties of Feline Leukemia Virus. III. Analysis of the RNA. Journal of Virology Vol. 16(3):535-545.

Step by step purification procedure:

1) Approximately 10 to 15 liters of harvested and clarified culture medium is concentrated at 4°C by membrane filtration using a Millipore-Pellicon Membrane Filter Unit with an exclusion limit of 100,000 molecular weight. Final concentration volume varies from 800 to 1,000 ml.
2) The concentrated culture medium is subjected to centrifugation at 25,000 rpm for 1 hour at 4°C in the Beckman SW 27 rotor to concentrate the virus and exclude it from other high molecular weight compounds concentrated from the medium (e.g. serum proteins).
3) The viral pellets are resuspended in 1X TNE buffer, pH 7.4 (0.01M Tris, 0.1M NaCl, and 0.001M EDTA) at 0°C. (From the original 15 liters, 12 pellets are obtained and resuspended in 5 ml of 1X TNE per pellet.)
4) The resuspended viral pellets are then layered onto 20-50% linear sucrose density gradients prepared in 1X TNE, pH 7.4 and centrifuged at 25,000 rpm for 1 hour at 4°C in the SW 27 rotor. (Five ml virus suspension is layered onto each of 12 30 ml gradients).
5) The dense viral bands which sediment approximately halfway down in the gradients are harvested manually from above with a long needle and syringe against a background of reflected light.
6) The combined viral fractions (approximately 150 ml) in sucrose are dialyzed at 4°C against 1X TNE, pH 7.4. Two changes of 6 liters each at 1 day intervals are sufficient to reduce the sucrose concentration to less than 1%.

7) The dialyzed virus material is finally concentrated by centrifugation at 25,000 rpm at 4°C in the SW 27 rotor for 1 hour.

8) The virus pellets are suspended in a total of 15 ml 1X TNE, pH 7.4 buffer and stored frozen at -80°C for future RNA extractions.

f. RNA extraction and purification procedures from Feline Leukemia Virus - KT Strain.

These methods are based on those presented in the following references:

1) Sherr, C.M., Fedele, L.A., Oskarsson, M., Maizel, J. and Vande 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. Journal of Virology, Vol. 34(1):200-212.

2.) Thomason, A.R., Brian, D.A., Velicer, L.F. and Rottman, F.M. 1976. Methylation of High-Molecular-Weight Subunit RNA of Feline Leukemia Virus. Journal of Virology Vol. 20(1):123-132.

3) Brian, D.A., Thomason, A.R., Rottman, F.M. and Velicer, L.F. 1975. Properties of Feline Leukemia Virus. III. Analysis of the RNA. Journal of Virology Vol. 16(3):535-545.

All glassware and buffers used are made nuclease-free by treatment with diethyl pyrocarbonate (Sigma). All steps are carried out at room temperature unless otherwise indicated.

1) The FeLV-KT in 1X TNE, pH 7.4 is made 1.0% with SDS by adding one-tenth volume 10% SDS in TNE, pH 7.4.

2) An equal volume of Proteinase K (Boehringer Mannheim; self-digested at 60°C for 20 minutes) at 500 ug/ml in 1X TNE, pH 7.4 is added such that the final proteinase K concentration is 250 µg/ml. This mixture is incubated for 30 minutes at 37°C.

3) Two volumes of freshly prepared 1X TNE, pH 7.4 - saturated phenol are added and the mixture is stirred slowly at room temperature for 30 minutes - 1 hour.

4) The mixture is centrifuged at 1,800 rpm for 10 minutes to separate the upper aqueous phase and lower, more dense phenol fraction. The aqueous layer is removed with nuclease-free pasteur pipets.

5) The aqueous phase is subjected twice more to steps 3 and 4. (Three phenol extractions in total.)

6) The final aqueous phase is made 0.2M with Na acetate by addition of one-tenth volume of 2M stock Na acetate (nuclease-free).

7) Two volumes of cold absolute ethanol are added and the RNA is precipitated by storage at -20°C for at least 24 hours or at -12°C for 3 to 5 days to assure maximum precipitation.

8) The precipitated RNA is collected by centrifugation at 5,000 rpm for 2 hours in the SS 34 rotor of the Sorval centrifuge at 0°C.

9) The RNA pellets are carefully decanted, drained and resuspended in as small a volume of 1X TNE, pH 7.4 as possible (approximately 200 to 300 µl per pellet).

10) The RNA is then layered onto 15-30% linear sucrose (nuclease-free) gradients prepared in 1X TNE, pH 7.4. Approximately 300 µl are layered onto 11.2 ml gradients. Centrifugation is at 38,000 rpm for 3.75 hours in the Beckman SW 41 rotor at 25°C.

11) Gradients are fractionated and scanned with an ISCO density gradient fractionator and UV monitor.

12) The peak fractions representing the viral 70 S RNA are collected and the RNA is reprecipitated by adding one-tenth volume of 2.0M Na acetate and two volumes of absolute ethanol, and storing at -22°C.

13) The precipitated 70 S viral RNA will then be collected as in step 8 and subjected to a second cycle in sucrose gradients (steps 8-12 are repeated a second time).

g. Proposed methods for preparing complementary DNA (C-DNA) to the 70 S Feline Leukemia Virus - KT strain RNA.

The following methods are based on the procedures of:

1) Thomason, R.R., Friderici, K.H., Velicer, L.F. and Rottman, F. 1978. Presence of 5'-Terminal Cap Structures in Virus-Specific RNA From Feline Leukemia Virus-Infected Cells. Journal of Virology 26(2):226-235. For optimal conditions for maximum yields of C-DNA to FeLV-Rickard strain 28S subunit RNA.

2) Taylor, J.M., Illmensee, R. and Summers, J. 1976. Efficient Transcription of RNA into DNA by Avian Sarcoma Virus Polymerase. Biochimica et Biophysica Acta 442:324-330. For preparation of oligonucleotide primer mixture from calf thymus DNA.

3) Retzel, E.F., Collet, M.S. and Faras, A.J. 1980. Enzymatic Synthesis of Deoxyribonucleic Acid by the Avian Retrovirus Reverse Transcriptase In Vitro: Optimum Conditions Required for Transcription of Large Ribonucleic Acid Templates. Biochemistry 19:513-518. For termination of the C-DNA reaction.

4) Collett, M.S. and Faras, A.J. 1975. Elimination of Residual RNase Activity from Purified Preparations of RNA-directed DNA Polymerase. Biochemical and Biophysical Research Communications. Vol. 67(3):946-955. For use of bentonite in elimination of RNase contamination.

5) Sherr, C.J., Fedele, L.A., Donner, L. and Turek, L.P. 1979. Restriction Endonuclease Mapping of Unintegrated Proviral DNA of Snyder-Theilen

Feline Sarcoma Virus: Localization of Sarcoma-Specific Sequences. Journal of Virology 32(3):860-875. For preparation of 32_p-labeled C-DNA.

A total reaction volume of 1 ml will be used and the reaction will be run in siliconized, nuclease-free 10 x 75 mm culture tubes, or in nuclease-treated 1.5 ml capacity microcentrifuge tubes (Walter Sarstedt, Inc).

The following reaction components are prepared as concentrated stocks distilled water which has been treated to remove nucleases (exception of calf thymus primers and RNA template - see ahead). They are added in the following amounts and in the following order:

	Stock Concentration	Amt added per 1 ml reaction volume	Final Concentration
1) Tris - Hcl, pH 8.49	900 mM	50 µl	50 mM
2) 2-Mercaptoethanol	2 M	10 µl	20 mM
3) $MgCl_2$	60.0 mM	50 µl	8 mM
4) KCl	1.0 M	50 µl	50 mM
5) dATP dGTP dTTP	10.0 mM each in a combined mixture.	10 µl of combined mixture.	1 mM 1 mM 1 mM
6) *Calf Thymus DNA primers (in 500 ul of 10 mM Tris-HCl, pH 7.4 + 10 mM $MgCl_2$)	5 mg/ml	500 µl	2.5 mg/ml
7) 70S FeLV-KT RNA Template (in 1X TNE, pH 8.3)	500 µg/ml	100 µl	50 µg/ml
8) Distilled H_2O containing Bentonite		300 µl	80 µg/ml of
9) **32_p-dCTP (New England Nuclear Corp: NEG 013X; Specific Activity 300 to 400 Ci/mmol).		100-200 µcl	

The above reaction mixture has a final pH of 8.3.

10) The reaction mixture is placed on ice for 5 minutes and Avian Myeloblastosis Virus Reverse Transcriptase (Biological Carcinogenesis Branch, National Cancer Institute,

Bethesda, MD) is rapidly added to a final concentration of 750 units/ml. (A stock solution of 7,500 units/ml is prepared and 100 µl is added to the reaction mixture.) One unit of enzyme activity is expressed as the incorporation of one nanomole of DTMP into an acid-insoluble product in 10 minutes at 37°C.

This mixture is quickly vortexed and incubated for 3 hours at 37°C. A time course will be done to determine if a longer incubation period is necessary to obtain a full-length copy.

Appropriate control mixtures will also be incubated under the same conditions as the C-DNA reaction mixture. Controls include:

1) All the above components minus RNA template.
2) All the above components minus the calf thymus DNA primer oligonucleotides.

* See details for the preparation of calf thymus DNA primers which follow on Pg. 8.

** 32_p dCTP will be used to generate a labeled complementary DNA product for hybridization to the cellular DNA obtained from the co-carcinogenesis transformation experiments.

Preliminary experiments will be done using unlabeled dCTP at a final concentration of 1 mM in a mixture with unlabeled dATP, dGTP, and dTTP also at 1 mM. The unlabeled C-DNA product will be used in reassociation experiments with ^{3}H-labeled FeLV RNA to determine if full-length copies of the viral genome have been generated under the reaction conditions used.

The reaction used to generate the Calf Thymus DNA primer mixture is as follows:

Oligonucleotides are generated by digestion of 5 mg of calf thymus DNA (Sigma) with 70 µg DNase I (Sigma) per ml in a reaction containing 10 mM Tris-HCl, pH 7.4 and 10 mM $MgCl_2$ at 37°C for 2 hours. Afterward the DNase I is inactivated by heating at 121°C for 10 minutes. The initial DNA concentration is used as the measure of the primer concentration. (Active primers in

such a mixture are approximately 8-15 nucleotides in length - Goulian et al., Biochemistry 12:2893, 1973.)

Preparation of Bentonite was according to that of: Garrett, C.T., Wilkinson, D.S. and Pitot, H.C. 1973. Preparation of Ribonuclease-free DNase I and a-Amylase. Analytical Biochemistry 52:342-348.

Following the enzymatic synthesis of C-DNA the reaction mixture is terminated by deproteinization with 0.5% SDS and Pronase or Proteinase K at 250 μg/ml final concentration at 37°C for 30 minutes. The mixture is then phenol extracted and subjected to either alkaline (0.3M NaOH for 4 hours at 37°C in STE-0.10M NaCl, 0.02M Tris-HCl, pH 7.4 and .001M Na_2EDTA) or RNase (40 μg/ml for 45 minutes at 37°C) hydrolysis to remove the template RNA. The C-DNA samples are then precipitated with absolute ethanol at -12°C and are either stored as such, or concentrated by centrifugation and suspended in an appropriate buffer volume for hybridization to the DNA from D 550 cells used for co-carcinogenesis transformation with various compounds.

Procedure which will be used to determine if full-length DNA copies have been transcribed from the FeLV-RNA template:

FL-74 cells which continuously shed Feline Leukemia virus KT strain will be grown in culture as described previously. ^{3}H-Uridine will be added to the media at a final concentration of at least 1 μCi/ml on day 5 for continuous labeling until the cells are harvested on day 8. (Reference: Brian, D.A., Thomason, A.R., Rottman, F.M. and Velicer, L.F. 1975. Properties of Feline Leukemia Virus. III. Analysis of the RNA. Journal of Virology 16(3):535-545.)

The medium will be clarified by centrifugation at 5,000 rpm for 15 minutes. Virus will be precipitated from the clarified medium in 10% polyethylene glycol (mol. wgt. 6,000, Sigma) and 0.5M NaCl. This mixture is stirred at room temperature for at least 1 hour and then placed at 4°C to stir for at least 36 hours.

The precipitated virus is collected by centrifugation at 5,000 rpm for 30 minutes and suspended in 1X TNE, pH 7.4 buffer.

This virus preparation is then further purified according to steps 4-8 in the section on FeLV virus purification on pages 13 and 14. The ^{3}H-labeled FeLV RNA (70 S) will be extracted and purified as described on pages 15, 16 and 17. This ^{3}H-labeled FeLV RNA will be hybridized to an excess of unlabeled C-DNA. S-1 nuclease will be used to digest any single-stranded nucleotides of the ^{3}H-RNA which remain unhybridized in the reaction (see procedures on following pages). Any loss of label from the hybridized product will be an indication that the full-length synthesis of the C-DNA was interrupted. Further steps will then need to be taken to improve the synthesis reaction or the reaction may be modified to prepare C-DNA to the subunit RNA (35 S) of FeLV rather than the complete genome (70 S) which consists of 2 identical linked subunits.

If a full-length C-DNA copy has been obtained, the kinetics of hybridization of ^{3}H-FeLV RNA to C-DNA should follow closely those of the ^{3}H-FeLV RNA to the unlabeled FeLV RNA also.

h. Proposed procedures to be used for hybridization reactions and enumeration of integrated FeLV genomes in D 550-infected cells treated with co-carcinogens.

The hybridization methods outlined by Maxwell et al. (Reference: Maxwell, I.H., Van Ness, J. and Hahn, W.E. 1978. Assay of DNA-RNA Hybrids by S-1 Nuclease Digestion and Adsorption to DEAE-Cellulose Filters. Nucleic Acids Research 5(6):2033-2038) will be followed closely.

S-1 nuclease-specific digestion of single-stranded DNA's or RNA's will be used to quantitate hybridization, and the S-1 resistant nucleic acid will be collected on filter discs of DEAE-cellulose which bind the hybrids and can be

efficiently washed free of digested nucleic acid. References for the use of S-1 nuclease include the following:

1) Ando, T. 1966. A Nuclease Specific for Heat-Denatured DNA Isolated from a Product of Aspergillus Oryzae. Biochimica et Biophysica Acta 114:158-168.

2) Benveniste, R.E., Heinemann, R., Wilson, G.L., Callahan, R. and Todaro, G.J. 1974. Detection of Baboon Type C Viral Sequences in Various Primate Tissues by Molecular Hybridization. Journal of Virology 14:56-67.

3) Sutton, W.D. 1971. A Crude Nuclease Preparation Suitable for Use in DNA Reassociation Experiments. Biochimica et Biophysica Acta 240:522-531.

S-1 nuclease preparation.

1.1×10^6 units of lyophilized S-1 nuclease (Miles Laboratories) will be dissolved in 0.44 ml H_2O and mixed with 1.1 mg bovine serum albumin (dissolved in 0.11 ml H_2O) and 0.55 ml glycerol. This preparation is stored at $-20^{\circ}C$.

DNA to RNA and DNA to DNA hybridization:

Hybridization reactions will be performed in nuclease-treated microcentrifuge tubes (capacity 1.5 ml) and overlayered with sterile mineral in order to prevent evaporation. Hybridizations will either be performed in:

1) 2 X SSC buffer (SSC = 0.15M NaCl, 0.015M Sodium Citrate) at $67^{\circ}C$. Reference: Misra, V., Muller, M.T., and Hudson, J.B. 1977. The Enumeration of Viral Genomes in Murine Cytomegalovirus-Infected Cells. Virology 83:458-461.

2) or in 0.01M Tris-hydrochloride (pH 7.6), 1 mM EDTA, 0.05% sodium dodecyl sulphate, and 0.65M NaCl at $68^{\circ}C$. Reference: Sherr, C.M., Fecele, L.A., Oskarsson, M., Maizel, J. and Vande 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. Journal of Virology 34(1):200-212.

Preliminary incubations will be done to determine the Cot (mole x sec/liter) values required for optimum reassociation of the nucleic acid species.

Hybrids will be assayed by diluting 1-5 µl in 100 µl of S-1 nuclease buffer (0.25M NaCl, 1 mM Zn acetate, 0.03M Na acetate, pH 4.5). To avoid changes in pH, and the inhibition of phosphate on S-1 nuclease, the dilution of the reaction mixture should be such that the phosphate concentration is less than or equal to 4 mM.

Sample sizes of 20-45 µl of the diluted mixture will be immediately applied to 2.5 cm DE-81 cellulose filter discs (Whatman) to determine total radioactivity per unit volume.

S-1 nuclease will then be added to the remaining volume at a concentration of 5,000-10,000 units/ml and this mixture will be incubated at 37°C for 80 minutes. After incubation 45-75 µl aliquots will be applied to DE-81 discs. All pipetting will be done with a calibrated Rainen pipet. Filters will be washed in small beakers for 10-15 minutes in 3-4 changes of 0.5M phosphate buffer with stirring, followed by 2 one-minute washes with water and one wash with 95% ethanol.

Filters will then be thoroughly dried, placed in liquid scintillation vials containing scintillation mixture, and counted in a Beckman liquid scintillation counter.

After preliminary reassociation reactions have been completed between ^{3}H-FeLV RNA and C-DNA to determine if full-length copies of C-DNA have

been obtained, the synthesis of ^{32}p-labeled C-DNA will be performed. The ^{32}p-labeled C-DNA will be used in hybridization reactions with cellular DNA extracted from D 550 cells infected with FeSV-ST and treated with co-carcinogens.

The enumeration of integrated FeSV genomes in cellular DNA

Procedures to be used are based on the methods of:

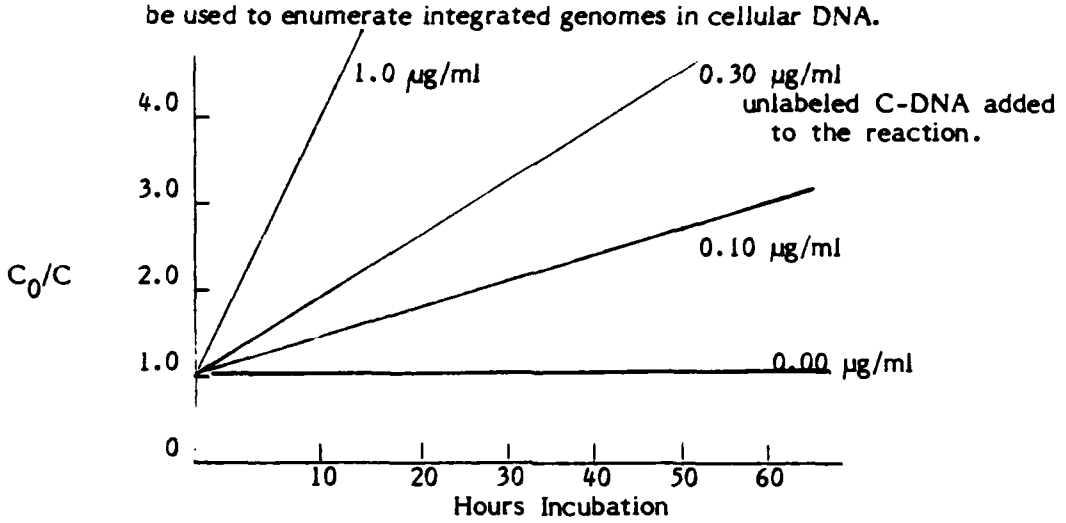
1) Misra, V., Muller, M.T. and Hudson, J.B. 1977. The Enumeration of Viral Genomes in Murine Cytomegalovirus-Infected Cells. Virology 83:458-46.1
2) Sugden, B., Phelps, M. and Domoradski, J. 1979. Epstein-Barr Virus DNA is Amplified in Transformed Lymphocytes. Journal of Virology 31(3):590-595.

These methods are based on the principle that the rate of reassociation of ^{32}p-labeled C-DNA is accelerated by unlabeled FeLV-C-DNA and the extent of this acceleration is proportional to the mass of the unlabeled C-DNA. The kinetics of the C-DNA to C-DNA reassociation follow the equation $C_0/C = 1 + k\ C_0T$ where C_0 = the initial concentration of single-stranded labeled C-DNA; C = the concentration of single-stranded labeled C-DNA at time t; and k = the reassociation constant. Reference: Britten, R.J. and Kohne, D.E. 1968. Repeated Sequences in DNA. Science 161(3841):529-540.

A plot of C_0/C versus time results in a straight line with the slope directly proportional to the concentration of unlabeled C-DNA added to the reaction mixture if the reaction follows second-order kinetics. Standard reconstruction kinetics as shown below will be plotted initially using a computer program and linear regression analysis.

Example of standard reconstruction plot analysis. A standard amount of labeled ^{32}p C-DNA is hybridized in the presence of various amounts of

unlabeled C-DNA to generate standard curves. The slopes of these lines will be used to enumerate integrated genomes in cellular DNA.



The hybridization reactions for the reassociation of ^{32}p-labeled FeLV C-DNA in the presence of unlabeled FeLV C-DNA will be done using ^{32}p-labeled FeLV C-DNA at a known concentration (4 ng/ml, at known cpm), in the presence of various concentrations of unlabeled FeLV C-DNA; e.g. 0.00 µg/ml, 0.10 µg/ml, 0.20 µg/ml, 0.50 µg/ml, 1.0 µg/ml, etc. where the concentration of unlabeled FeLV C-DNA in each reaction mixture is adjusted to 500 µg/ml with calf thymus DNA.

Once the slopes have been calculated, these reconstruction plots can be used to determine the absolute quantities of viral DNA in infected D 550 cells and to calculate genome equivalents. Such calculations are based on:

1) The molecular weight of the FeLV C-DNA genome, and its kinetic complexity being approximately equal to its molecular weight, and
2) The molecular weight of diploid mammalian DNA being 3.92×10^{12}.

Absolute molecular weight of FeLV C-DNA may be determined by the methods of Mosmann, T.R. and Judson, J.B. 1973. Some Properties of the

Genome of Murine Cytomegalovirus (MCV). Virology 54:135-149, using relative rates of sedimentation of FeLV C-DNA and T4-DNAs.

Prior to hybridization of ^{32}p-labeled FeLV C-DNA to cellular D 550 DNA, the cellular D 550 DNA will be sheared sonically to an average size of 5 S. Hybridization mixtures will be denatured in boiling water for 20 minutes, cooled rapidly, and transferred to a water bath equilibrated at 67°C. S-1 nuclease treatment and application to DE-81 cellulose disc filters will be done as described previously.

Results to date:

Co-carcinogenesis transformation experiments which have been done with the D 550 cells.

As indicated in the section on methods, the following co-carcinogens and administration times have been completed and the cells are frozen at -80°C.

1) Hydrazine administered both 2 hours pre- and post-infection with FeLV-ST.
2) Methyl hydrazine administered both 2 hours pre- and post-infection with FeLV-ST.
3) D 550 normal control cells (no compound or FeLV-St administered).
4) D 550 cells infected only with FeLV-ST (no co-carcinogen treatment).

Experiments which remain to be done are:

1) Symmetrical and unsymmetrical dimethyl hydrazine at 6 hours pre- and post-infection with FeLV-ST.
2) Administration of Benzo(a)pyrene 2 hours post-infection (in progress) and 2 hours pre-infection with FeLV-ST.
3) Administration of Pyrene (non-carcinogenic analogue of Benzo(a)pyrene) at 2 hours pre- and post-infection with FeLV-ST.

Extraction of cellular DNA

Was done with normal D 550 cells to verify the adequacy of the extraction procedure.

The other DNA's from co-carcinogen treated D 550's will be extracted shortly before the time of the final hybridization reactions to prevent degradation on storage.

Purification of FeLV-KT from FL-74 cells

Weekly or biweekly purifications of 15 liters of culture medium have been made to build up a stock of virus for RNA extraction. A substantial amount of virus has been stored frozen at -80°C and used for RNA extraction and more virus will be grown and purified as needed.

FeLV-KT 70 S RNA extraction and purification

Adequate amounts of 70 S RNA to be used in the C-DNA reaction are being reproducibly purified by density gradient centrifugation. Following is a profile of the 70 S RNA species of 15-30% linear sucrose density gradients centrifuged for 3.75 hours at 38,000 rpm at 25°C in the SW 41 rotor (Beckman) (Fig. 1). Gradients were scanned at 254 nm wavelength. Note the highly absorbing material at the top of the gradient followed by the 35 S subunit RNA species, and finally the intact 70 S RNA species of interest in the bottom one-third of the gradient. Size determinations were made on the basis of 16 S and 23 S markers from E. coli subjected to identical centrifugation conditions. The aggregate of the 16 S and 23 S markers sediments in almost the same position as the 35 S subunit species of the FeLV-ST. The 70 S RNA species is being collected and reprecipitated in absolute ethanol at -20°C for use in preparing the C-DNA.

Amounts of 70 S necessary to begin the C-DNA synthetic reactions have been recovered.

FL-74 cells are being grown for labeling of the FeLV-RNA with ^{3}H-uridine. This labeled RNA will be used in determining the adequacy of the C-DNA reaction in fully copying the FeLV-RNA genome.

Detroit 550 cells are being continually grown in culture and used for the co-carcinogenesis transformation experiments to obtain DNA for hybridization to ^{32}p-FeLV-C-DNA.

Methods are still being worked out for the synthesis of complementary DNA and its final hybridization to D 550 cellular DNA species from co-carcinogenesis experiments to determine the effects of the carcinogen on viral transformation and genome integration into human cellular DNA.

C. Results of experimentation (continued)

3. Effects of acetone on virus transformation assay system. 2% and 0.2% v/v acetone significantly inhibited transformation when cells were exposed 24 hours and 6 hours (2% only) before infection (Fig. 2). No significant inhibition resulted when acetone treated cells were infected at +2, +6, or +24 hours p.i. Data suggest acetone non-carcinogen.
4. Effects of formalin on virus transformation. Ten and 1.0 ppm showed dose-related effects (Fig. 3). Cells treated with 10 ppm resulted in significant inhibition at -24, -6, -2, and +2 hours in relation to virus infection. Low-dose (1.0 ppm showed slight reduction in transformation at -24 hours only. Data suggest formalin is carcinogenic.
5. Effect of petroleum derived (Pet) and shale oil derived (SOD) JP 5 jet fuels on transformation assay system: Both fuels were tested at 2 dose levels (50. and 5.0 ppm). Both doses of SOD JP 5 inhibited transformation at -24 hours only (Fig. 4). No effect with JP 5 (Pet) at either concentration or at 6 time periods affected transformation. The data suggest JP 5 non-carcinogenic.
6. Effect of Triton-X-100 on virus transformation: Triton-X-100 was used in attempts to solubilize jet fuels for cell treatment. As shown in Fig. 5, no inhibition resulted after treatment with 2 ppm or 0.2 ppm. However, 2 ppm showed slight enhancement at 2 time periods.
7. SDMH - Disulfiram Experiments - Disulfiram has been shown to inhibit SDMH-induced colon carcinogenesis in mice. These experiments were designed to test

the specificity of the antagonistic effect of SDMH on virus transformation. Co-treatment with SDMH and disulfiram should abrogate the anti-carcinogenic (antagonistic) effect of SDMH on virus transformation. As shown in Table 1, SDMH significantly inhibited ST FeSV transformation. Co-treatment (3) resulted in no significant difference in transformation frequency when compared to the controls. Thus, the antagonistic effect of SDMH on virus transformation was abrogated by disulfiram.

8. Disulfiram inhibits biotransformation of SDMH metabolites azomethane to azoxymethane, thereby preventing the carcinogenic effect of parent SDMH. In Table 2, MAMA (1 ppm) reduced the transformation frequency by 41% whereas MAMA (1 ppm) and disulfiram (0.1 μg/ml) reduced the frequency by 37%. Thus, the antagonistic effect of MAMA on virus transformation was not abrogated by disulfiram.

The date presented in 7 and 8 provide indirect evidence for specificity of virus transformation assay system.

9. Preliminary experiments with ^{14}C SDMH have been performed to determine certain parameters such as cell number, DNA isolation techniques, concentration of isotope used to label DNA. Table 3 depicts these preliminary results. Detectable levels of isotope labeled DNA were found for both SDMH and UDMH. Co-treatment with ST-FeSV and UDMH and SDMH resulted in a greater labeling than did the addition of either chemical alone. Disulfiram did not inhibit the labeling of cellular DNA by SDMH as predicted. Possibly the ratios of concentration of disulfiram (0.01 μg/ml) to SDMH (100 μg/ml) will have to be altered to effectively prevent the biotransformation of SDMH to its ultimate carcinogen methylazoxymethanol.

10. Table 4 is a compilation of all the chemicals tested in this system and the

correlation between known carcinogenic and non-carcinogenic compounds. Footnote in table indicates sources of information for carcinogenic compounds.

III. Written Publications (See Appendix)

1. Blakeslee, J.R., Elliott, A. and Turner, D. (1980) Induction of Retrovirus Non-Producer Human Cells to Producer Cells by Dexamethasone. Adv. in Compar. Leuk. Res. 1979. Eds. D. Yohn, B. Lapin and J. Blakeslee. pp:87-88.
2. Blakeslee, J.R. (1981): Inhibition of Virus Transformation by High Energy Fuels as a Correlate of Carcinogenic Potential. AGARD Publication Conference Proceeding 309 pp. B6-1 - B6-3, Technical Editing and Reproduction Ltd. Harford House, London, England.
3. Blakeslee, J.R., Elliott, A. and D. Turner (1981): Enhanced Permissiveness of Human Cells to Feline Retrovirus Infection Following Hormone Treatment. In Adv. in Comp. Leuk. Res. Eds. B. Lapin and D. Yohn. pp:121-124. USSR Acad. Med. SCI/IEPET - Moscow.
4. Tarr, M. and Blakeslee, J.: Factors Affecting Feline Retrovirus Infectivity and Oncogenicity. In Press: Feline Leukemia, Ed. R. Olsen. CRC Press, Boca Raton, Fla. 1981.

IV. Professional Personnel Associated with Research Effort

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V. Oral Presentations

1. Aerospace Medical Panel
   Specialists Meeting, Toronto, Canada
   September 15-19, 1980.

2. Review of AF Sponsored Basic
   Research in Environmental Toxicology
   Columbus, Ohio
   June 2-3, 1981.

VI. Significance of Results to Date and Future Direction

To date, chemicals of known carcinogenic activity significantly antagonized ST-FeSV virus transformation of human cells, whereas non-carcinogens did not.

The antagonistic effect of 1,2 dimethylhydrazine on virus transformation was abrogated by disulfiram.

The antagonistic effect of methylazoxymethanol acetate on virus transformation was not abrogated by disulfiram.

Disulfiram inhibits biotransformation of 1,2 dimethylhydrazine metabolites azomethane to azoxymethane, thereby preventing carcinogenicity of parent compound.

Indirect evidence for specificity of virus transformation assay system has been provided by this evidence. Future directions of this research will be to furher develop the antagonistic virus assay with chemical carcinogens and non-carcinogenic analogues; to determine the mechanisms involved by hybridization analysis and other radiochemical techniques and to determine whether carcinogenic chemicals affect virus gene expression (P28) in a predictable quantitative assay.

VII. No Animals Were Used in These Studies

VIII. Appendix

FIGURE 1.

O.D. 254 nm PROFILE OF PURIFIED KT FeLV RNA ON A

15%-30% LINEAR SUCROSE DENSITY GRADIENT.

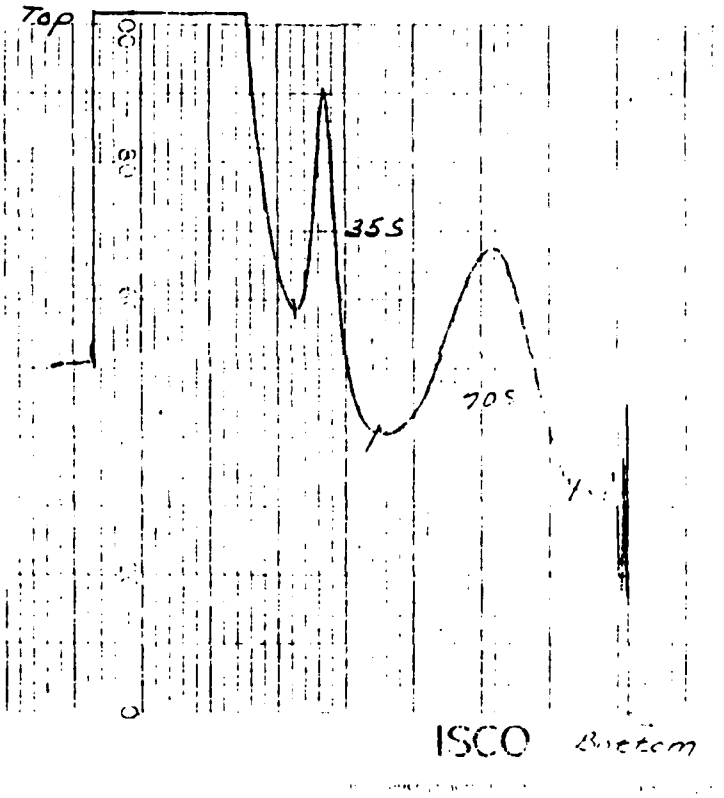


FIGURE 2.

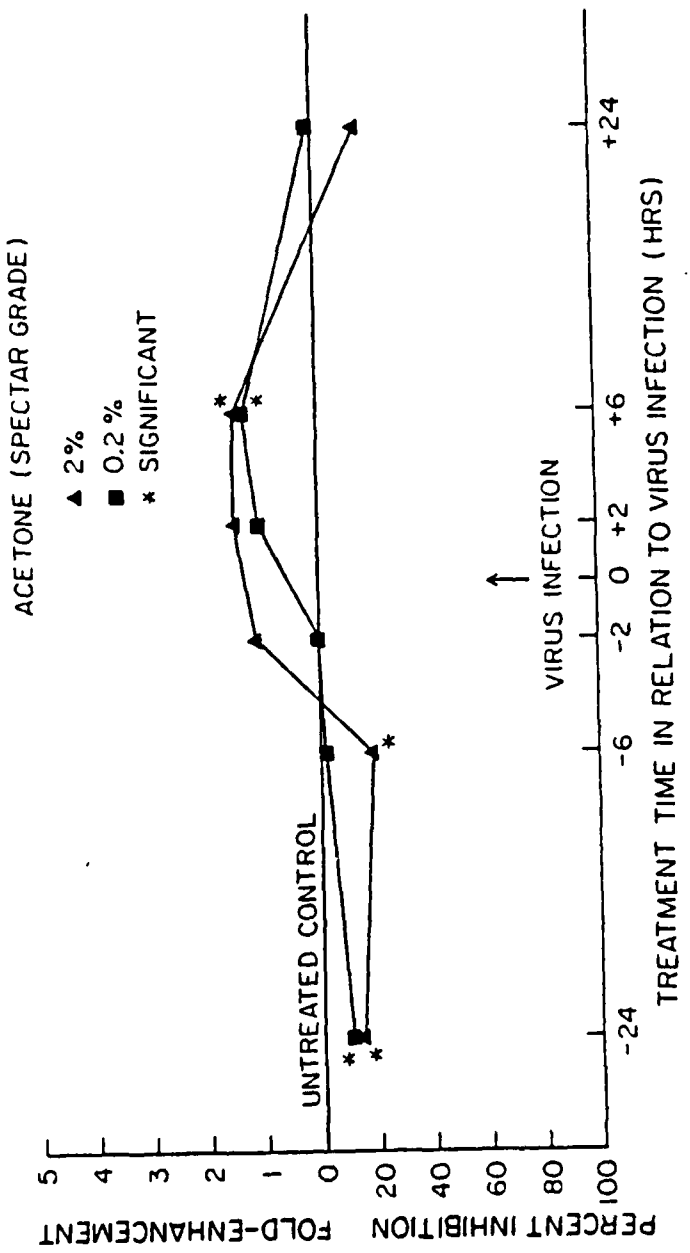
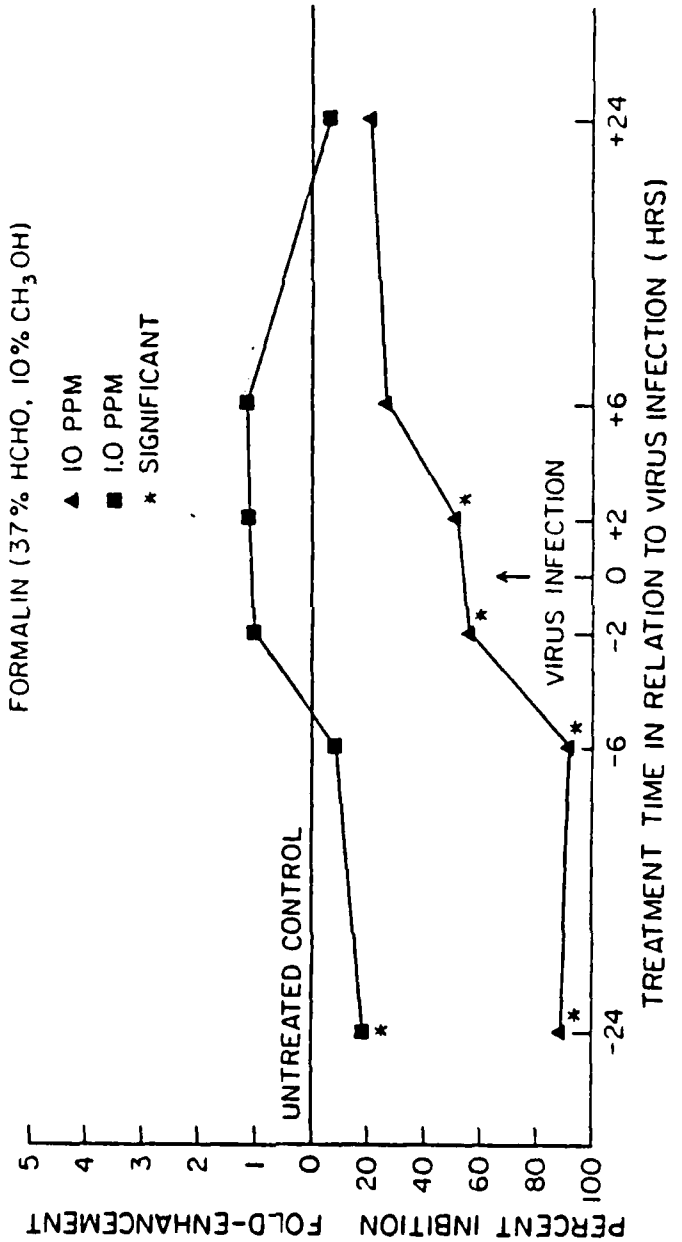

ACETONE (SPECTAR GRADE)
▲ 2%
■ 0.2%
* SIGNIFICANT
UNTREATED CONTROL
VIRUS INFECTION
FOLD-ENHANCEMENT
PERCENT INHIBITION
5
4
3
2
1
0
20
40
60
80
100
-24
-6
-2
0
+2
+6
+24
TREATMENT TIME IN RELATION TO VIRUS INFECTION (HRS)

FIGURE 3.


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

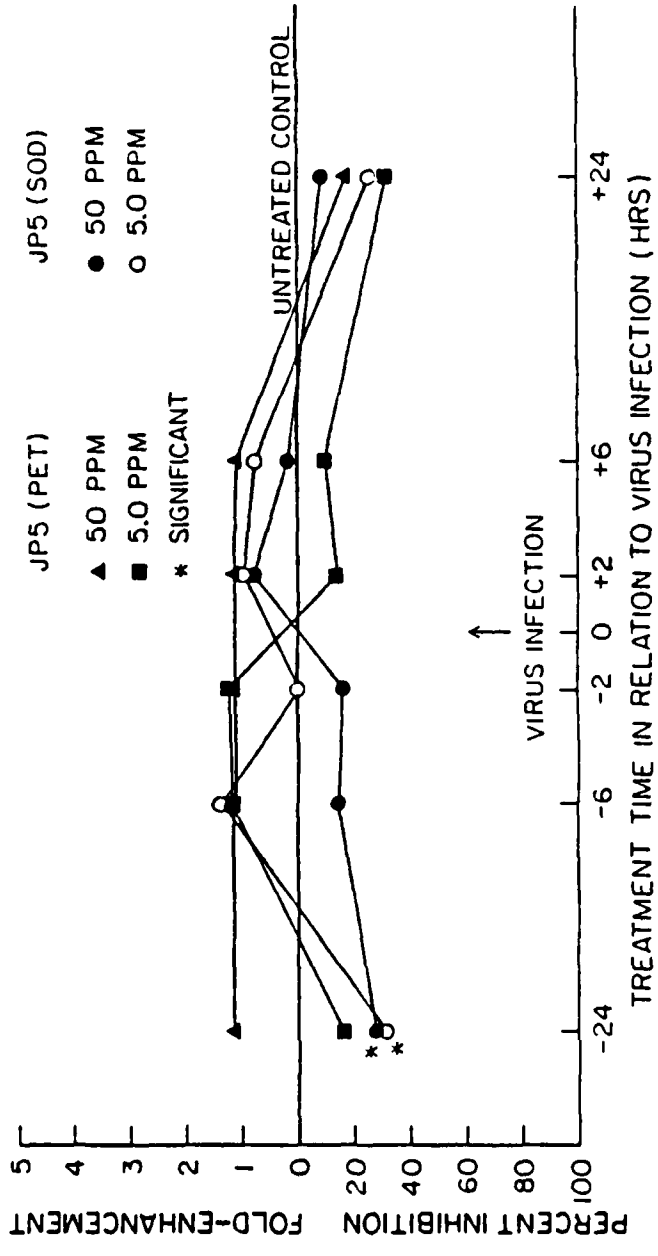

JP5 (PET)
▲ 50 PPM
■ 5.0 PPM
* SIGNIFICANT
JP5 (SOD)
● 50 PPM
o 5.0 PPM
UNTREATED CONTROL
VIRUS INFECTION
FOLD-ENHANCEMENT
PERCENT INHIBITION
TREATMENT TIME IN RELATION TO VIRUS INFECTION (HRS)
-24
-6
-2
0
+2
+6
+24
5
4
3
2
1
0
20
40
60
80
100

FIGURE 4.

FIGURE 5.

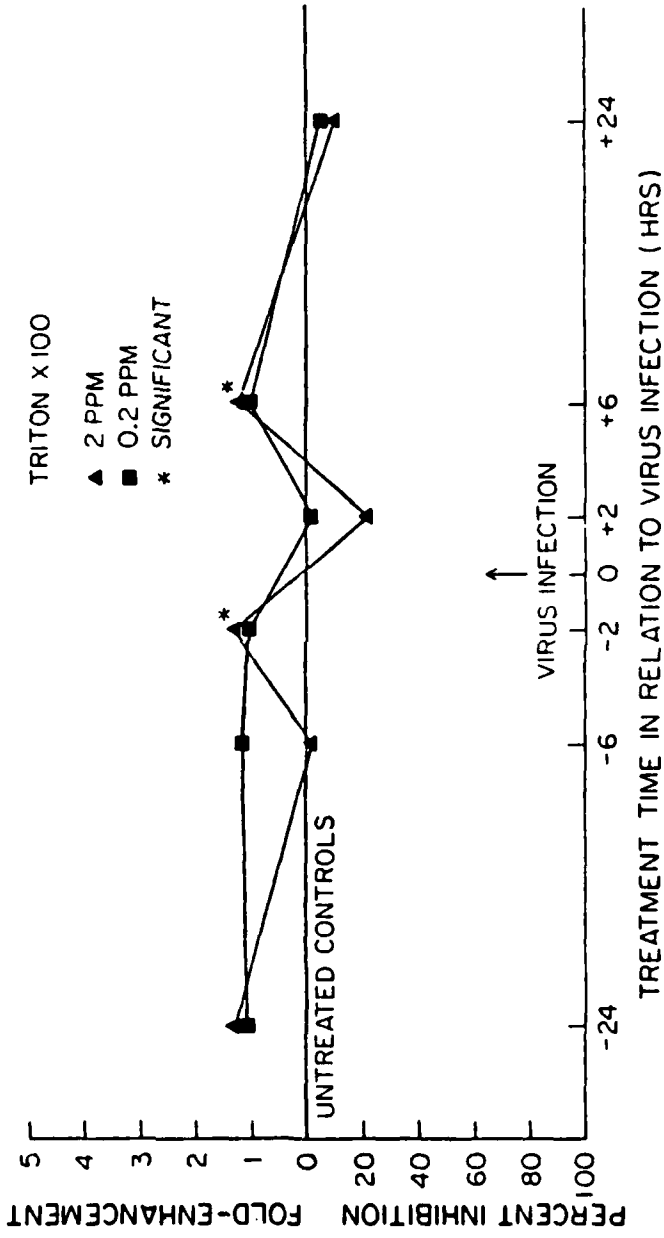

TRITON X100
2 PPM
0.2 PPM
SIGNIFICANT
FOLD-ENHANCEMENT
PERCENT INHIBITION
UNTREATED CONTROLS
VIRUS INFECTION
-24
-6
-2
0
+2
+6
+24
TREATMENT TIME IN RELATION TO VIRUS INFECTION (HRS)

Table 1.

Abrogation of the Anti-Carcinogenic Effect of 1,2 Dimethylhydrazine On ST-FeSV Transformation by Disulfiram

Chemical Treatment	Virus Titer FFU/ml ($X10^4$)	% Reduction in Titer	(P)[1]
1. ST-FeSV (control)	2.51	-	-
2. SDMH (10 ug/ml)	1.41	44%	0.001
3. SDMH (10 ug/ml) + DS (0.01 ug/ml)	1.96	22%	N.S.

[1](P) = determined by Student's t test.

Table 2.

Failure of Disulfiram to Abrogate the Anti-Carcinogenic Effect of Methylazoxymethanol (Acetate)

Chemical Treatment	Virus Titer FFU/ml (X 10^4)	% Reduction in Titer	(P)[1]
ST-FeSV	1.86	-	-
MAMA (1 PPM)	1.10	41%	0.050
MAMA (1 PPM) + DS (0.01 ug/ml)	1.19	37%	0.010

[1](P) = Determined by Student's t test.

Table 3. - DNA Labeling of Human Cells by ^{14}C-UDMH and ^{14}C-SDMH.

	Chemical* Treatment (conc.)	CPM/0.1ml	Total dpm
1.	^{14}C-UDMH (10 PPM)	1,386	36,960
2.	^{14}C-UDMH (10 PPM) + FeSV	3,805	101,493
3.	^{14}C-SDMH (100 µg/ml)	1,086	28,960
4.	^{14}C-SDMH (100 µg/ml) + FeSV	1,219	32,507
5.	^{14}C-SDMH (100 µg/ml) + Disulfiram (0.01 µg/ml) + FeSV	1,473	39,280

*Cells were treated 24 hours, washed 2X in PBS, scraped and frozen -85°C until DNA isolated.

Table 4. Correlation Between Inhibition of Virus Transformation and Diverse Chemical Carcinogens

Chemical Group	Reported Activity[1]	Inhibition of ST-FeSV Transformation
I. Aromatic Amines		
A. Napthyl Amines		
1. Two (2)	C	Yes
2. Phenyl-alpha	C	Yes
3. Phenyl-beta	C	Yes
II. Polycyclic Hydrocarbons		
1. Benzo(a)pyrene	C	Yes
2. Pyrene	NC	No
III.		
1. N-Acetoxy 2 fluorenyl-acetamide	C	Yes
IV. 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
V. Other		
1. Aflatoxin B_1	C	Yes
2. Amosite Asbestos	C	Yes
3. JP4 (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 - noncarcinogen

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

(2) "Origens 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. AMEL, WPAFB.

ADVANCES IN COMPARATIVE LEUKEMIA RESEARCH 1979.
EDITORS LAPIN ET AL
1980

ENHANCED PERMISSIVENESS OF HUMAN CELLS TO FELINE RETROVIRUS INFECTION FOLLOWING HORMONE TREATMENT

JAMES BLAKESLEE, ANN ELLIOTT AND DEBRA TURNER

Department of Veterinary Pathobiology, 1925 Coffey Road, Columbus, OH 43210, U.S.A.

We previously reported human neonatal skin fibroblast cells treated with non-toxic concentrations of benzo (a) pyrene or aflatoxin B, inhibited feline sarcoma virus (FeSV) transformation[1]. Although comparable levels of reverse transcriptase (RT) activity were found in both chemically treated and untreated cultures, infectious virus was not recoverable from either.

Others have documented the stimulation and activation of murine and primate retroviruses and enhanced FeSV transformation of human cells by optimal concentrations of glucocorticoid hormones[2-4].

The objectives of this study were to determine: 1) whether dexamethasone (DXM) induced both FeSV and FeLV synthesis in the apparent non-producer human cells and 2) whether feline oncornavirus-associated cell membrane antigen (FOCMA) and group-specific antigens (GSA) were synthesized in addition to RT, in non-DXM treated cells.

Snyder-Theilen strain FeSV[4] infected Detroit 550 neonatal human skin fibroblast cells produced very low levels of transforming virus when supernatant fluids from these cultures were titrated in normal D550 cells. The addition of 1.0 μg/ml DXM (9α-Fluoro-16α-methyl prednisolone) 24 hours post infection, significantly increased both FeSV and FeLV[5] synthesis when compared to non-DXM treated infected cells (Table 1). The increase in titer ranged from a 56-fold increase to a 100-fold increase for FeSV, while FeLV increased from 0 in untreated cultures to 6×10^4 FFU/ml in DXM treated cultures, a 2.6 fold excess when compared to FeSV.

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SCI/IEP&T - MOSCOW
Eds. B.A. LAPIN & D.S. YOHN. 1980.

Table 1

DXM enhanced ST-PeSV and PeLV synthesis in D550 cells

Virus and Exp #	Virus Titer PFU/ml X 10^{-2} (-) DXM	(+) DXM	Fold Increase
(1)	2.3	203.2	87X
(2)	2.3	233.0	100X
(3)	3.4	191.2	56X
Untreated Control	0	0	0
mean - PeLV[3]	2.7 ±.5 mean- 0	209.2 ±17.6 600.00	P = 0.001[a]

[a]Determined by Students "t" test.

RT activity (cpm of ^{3}H dTMP incorporated/reaction/hr)[1] likewise increased. The values ranged from a 9-fold increase in 1 experiment to a 11-fold increase in 2 other separate experiments.

GSA[6] and POCMA[7] were detected in both PeSV infected DXM treated cells and PeSV infected cells only. Uninfected-cells, cell treated with 0.2 % acetone, and/or DXM were negative (Table 2).

Table 2

POCMA and GSA antigen expression in ST PeSV infected cells

Cells Treated with[1]	GSA	POCMA
PeSV	+	+
PeSV + DXM	+	+
DXM	-	-
PeSV + Acetone[2]	+	+
Acetone	-	-
EMEM medium	-	-

[1]Cells examined 72 hours post infection.
[2]0.2 % v/v acetone in complete EMEM medium.

Wu et al.[8] described retrovirus infected cells as falling into 3 phenotypic categories with respect to virus gene expression: (1) virus producer cells; (2) non-producer cells with partial gene expression (any virus, component); and (3) cells not having any detectable viral gene products. In this study, D550 cells infected

with ST-FeSV could be classified as nonproducers with partial gene expression, i.e. transformation, reverse transcriptase, GSA and FOCMA antigens with little infectious virus being released. However, following DXM treatment, complete viral gene expression resulted with infectious (transforming) virus released.

The fact that DXM permits complete oncogenic viral gene expression in human cells is significant since the phenotypic expression of most human transformed cells has formerly been shown to be category 2 (non-producer) with partial gene expression.

ACKNOWLEDGEMENTS

Supported in part by a contract from AFOSR, F49620-77-C-011 and The Graduate School, The Ohio State University.

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2. Ahmed et al. (1977), J. Natl. Cancer Inst. 58, 1515-1518.
3. Wu et al. (1974), J. Virol. 14, 802-812.
4. Schaller et al. (1976), Cancer Res. 36, 1880-1887.
5. Heding et al. (1976), Cancer Res. 36, 1647-1652.
6. Hardy et al. (1973), in Unifying Concepts of Leukemia, Dutcher, R. and Chieco-Bianchi, L. ed., Karger, Basel pp. 778-799.
7. Essex et al (1971), Int. J. Cancer 8, 384-390.
8. Wu et al. (1976), in Bibl. Haematol. 43, 475-480.
9. Gallo R.C. (1973), Biomedicine 18, 446-452.

Yohn, Leoin, Blakeslee, eds. Advances in Comparative Leukemia Research 1979

INDUCTION OF RETROVIRUS NON-PRODUCER HUMAN CELLS TO PRODUCER CELLS BY DEXAMETHASONE

JAMES BLAKESLEE, ANN ELLIOT AND DEBRA TURNER.
Department of Veterinary Pathobiology, 1925 Coffey Road, Columbus, OH 43210, U.S.A.

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(2)	2.3	233.0	100X
(3)	3.4	191.2	56X
Untreated Control	0	0	0
mean -	2.7 ± .5	mean - 209.2 ±17.6	P = <0.001*
FeLV[3]	0	600.00	

* = Determined by Students "t" test.

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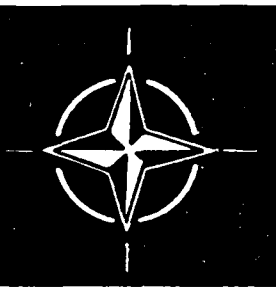

Inhibition of Virus Transformation by High Energy Fuels as a Correlate of Carcinogenic Potential

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Department of Veterinary Pathobiology, The Ohio State University Comprehensive Cancer Research Center, and the Department of Microbiology,
The Ohio State University, 1925 Coffey Rd., Columbus, Ohio 43210, U.S.A.

Summary

Hydrazine and naphthylamines and their derivatives were assayed for co-carcinogenic effects on ST FeSV-directed transformation of human cells. All chemicals tested at non-toxic concentrations showed anti-carcinogenic activity. The temporal relationship of chemical treatment to virus infection was more critical with the hydrazines than with the naphthylamines in that maximum anti-carcinogenic effect occurred when virus-infected cells were exposed to the hydrazines 2 hrs. post-infection, whereas the naphthylamines anti-carcinogenic effect was observed if cells were exposed either pre- or post-infection. The anti-carcinogenic effect, when compared with in vitro chemical *transformation and neoplastic transformation, show a high degree of correlation. These data suggest this* assay system may lend itself to a rapid screen (9-13 days) of chemicals for carcinogenic potential. Cytotoxic results showed no significant difference in shale oil or petroleum derived JP5 or DFM. Co-carcinogenic potential of JP5, JP10, RJ5, and DFM are being evaluated.

Introduction

Previous studies from this laboratory showed chemical carcinogens inhibited virus-directed feline sarcoma virus transformation of human skin fibroblast (HSF) cells at non-toxic concentrations (1). 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 carcinogens, 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 hrs. post-infection.

Hydrazine and its derivatives have widespread use in medicine, agriculture, and aerospace research (3). While many biologic effects of hydrazine (HZ) and its derivatives, monomethyl hydrazine (MMH), 1,1 dimethylhydrazine (UDMH), and 1,2 dimethylhydrazine (SDMH) have been studied in animals, extrapolation of these biologic effects to man has been difficult because of differential responses manifested in diverse species of test animals. Additionally, the different chemical properties of each of these chemicals has led to differential physiological responses within the same species. For example, Diwan et al. (4) concluded that genetic differences with inbred strains of mice affected the response of DMH carcinogenesis. Thus, carcinogenesis assays in rodents may lead to false negative results based on that genetic strain used in the assay. Ideally, economic, short-term, reliable in vitro assays would be invaluable in determining carcinogenic potential of chemicals.

In this study, we examined the co-carcinogenic effect of alpha-naphthylamine (ANA), phenyl-alpha naphthylamine (PANA), phenyl-beta naphthylamine (PBNA), HZ, MMH, UDMH, and SDMH on Snyder-Theilen feline sarcoma virus (ST-FeSV) transformation of HSF. The effects were further correlated with in vitro chemical transformation of HSF described by Milo and Blakeslee (3). Cytotoxic analyses of petroleum and shale oil derived jet fuels are also presented.

Materials and Methods

Cells

Human foreskin fibroblast cells (Detroit 550-CCL109, 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) 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 37 C in 5% CO_2.

Virus

The preparation of stock ST-FeSV was described previously (6). Briefly, 10% cell-free homogenates were prepared and frozen at -85C in L15 medium and 5% FBS.

Co-Chemical Virus Assays

Preconfluent log phase growth HSF cells were trypsinized and 4 X 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 anpropriate 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 ofDEAE-Dextran (40µg/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 post-infection (PI), and subsequently fixed with 10% phosphate buffered formalin and stained with Giemsa 3-4 days later. Foci were counted at 25-40X 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.

Chemicals

ANA, PANA, and PBNA were dissolved in spectral grade acetone at 1.0 mg/ml. Prior to use, dilutions were made in CM to experimental concentrations. HZ, MMH, and UDMH were pipetted into CM at 1 X 10^6 ppm and diluted 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 were dispersed in equal volumes of 20% Triton-X (Sigma, St. Louis, MO) serum-free CM with shaking. Dilutions tested were from 5000 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

Naphthylamines

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. post-infection 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 μg/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. post-infection.

Figure 1C shows the effect of 10 μg and 0.01 μg/ml ANA. Both concentrations used inhibited ST-FeSV transformation, with the most significant inhibition observed when cells were treated from 2-6 hrs. pre-infection. Cells treated 6 hrs. or 24 hrs. post-virus infection had no effect on virus transformation.

Hydrazines

MMH (100 ppm and 10 ppm) significantly enhanced virus transformation in a dose-dependent manner when cells were exposed 2 hrs. pre-infection (Fig. 2A). Conversely, treatment of virus-infected cells resulted in significant inhibition of transformation when cells were treated 2 hrs. or 6 hrs. post infection. No effect was noted at 24 hrs. post-infection.

SDMH (100 μg/ml and 10 μg/ml) likewise enhanced virus transformation when cells were treated 2 hrs. pre-infection (Fig. 2B). In virus infected cells, SDMH treatment inhibited transformation by values ranging from 20% (10 μg/ml) to 30% (100 μg/ml). The inhibitory effect was observed at 2 hrs. post-infection, but not at 6 or 24 hrs. post-infection.

The effects of UDMH on virus transformation are shown in Fig. 2C. One hundred or 10 ppm exposure to UDMH inhibited transformation at 3 different time periods in relation to virus infection. UDMH inhibited transformation by values ranging from 25% to 50% when cells were exposed 6 hrs. before or 2 hrs. and 24 hrs. post-infection.

The results of HZ (60 ppm and 6 ppm) exposure are shown in Fig. 2D. Fig. 2D is a composite figure of 3 separate experiments showing HZ effects on virus transformation. Like SDMH and MMH, cells exposed to HZ 2 hrs. pre-infection, significantly enhanced virus transformation whereas exposure 2 hrs. post-virus infection significantly inhibited virus transformation as did MMH, SDMH, and UDMH.

Cytotoxicity of Fuels

Petroleum derived (PD) or shale oil derived (SOD) JP5 or diesel fuel, marine (DFM) cytotoxic analyses are shown in Table 1. The results show no significant difference in toxicity values between SOD or PD fuels. LD_{50}'s for SOD, JP5 and PD-JP5 were 102 ppm and 100 ppm, respectively, SOD-DFM and PD-DFM were 85 ppm and 87 ppm. 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[1]	Shale	85
DFM	Petroleum	87
RJ5	Petroleum	19
JP10	Petroleum	91

[1] DFM = diesel fuel, marine

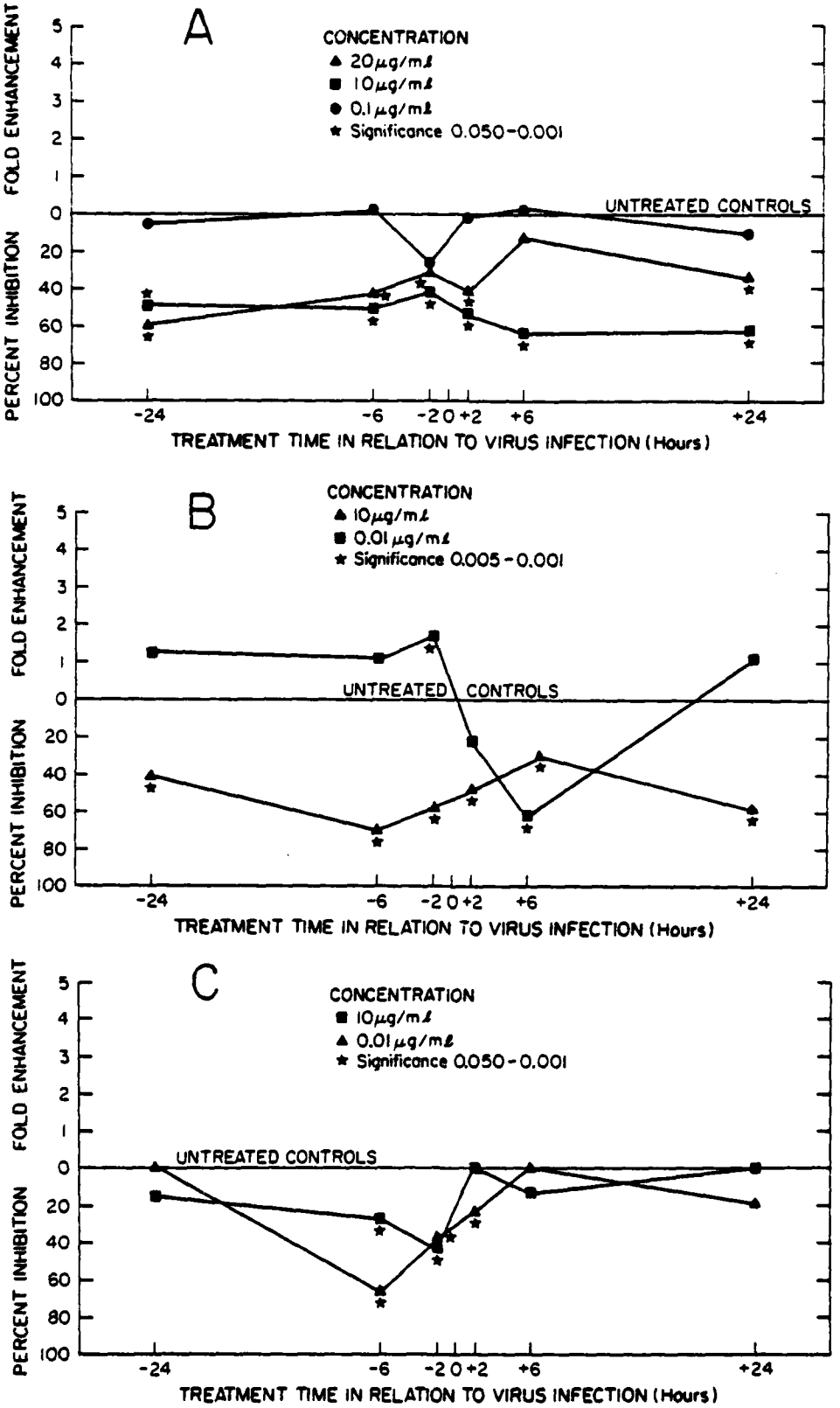

Fig. 1. HSF cells were plated in 16 mm diameter wells with 2.0 ml CM and incubated overnight. Cells were treated with ANA as described in Materials and Methods. (-) indicates cells treated before virus infection (hrs). (+) indicates cells treated after virus infection (hrs). * - significance determined by Student "t" test. A-PANA, B-PBNA and C-ANA.

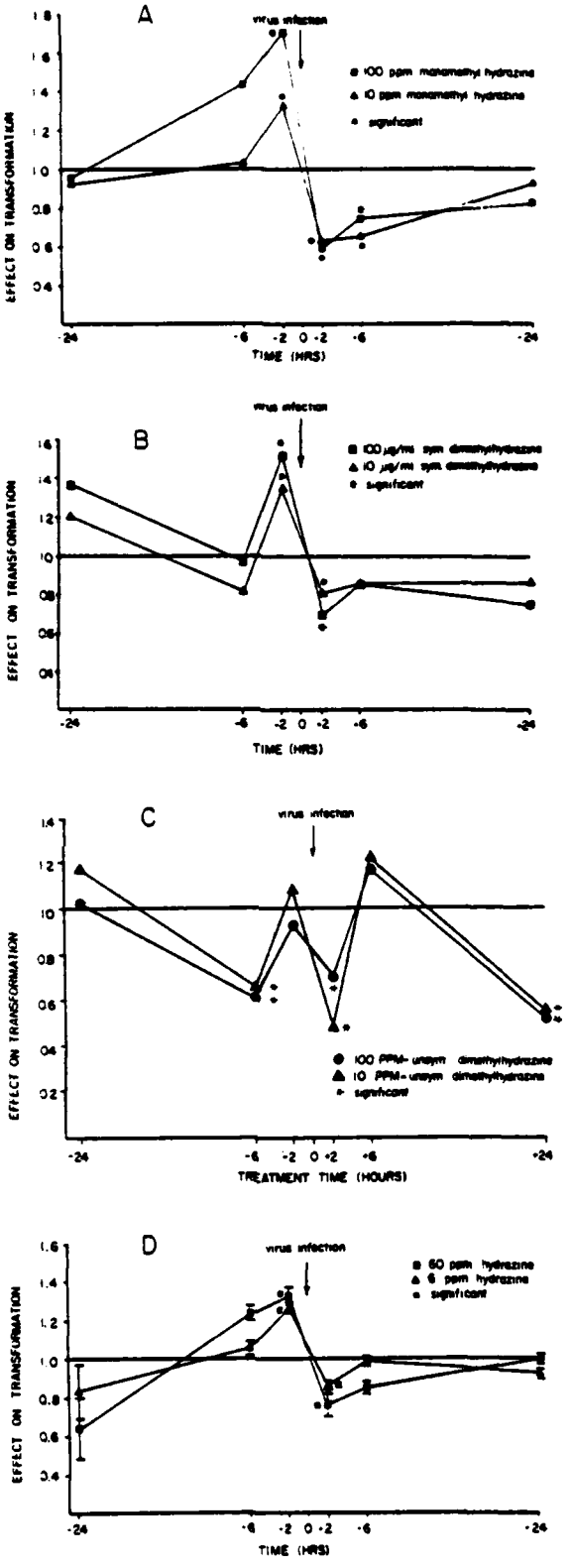

Fig. 2. HSF cells were plated in 16 mm diameter wells with 2.0 ml CM and incubated overnight. Cells were treated with ANA as described in Materials and Methods. (-) indicates cells treated before virus infection (hrs). (+) indicates cells treated after virus infection (hrs). * - significance determined by Student "t" test. A-MMH, B-SDMH, C-UDMH and D-HZ. D is a composite of 3 separate tests. Each point - n = 48 ± S.D.

Discussion

The co-carcinogenic effects of hydrazine and its derivatives and naphthylamines described in this report, when correlated with in vitro chemical transformation and neoplastic transformation, show a high degree of correlation (Table 2).

ANA, PANA, and PBNA showed 100% correlation with the two parameters, whereas MMH and SDMH showed activity in the co-carcinogenesis assays, but not in the carcinogenesis assays. HZ and UDMH, like the naphthylamines, showed 100% correlation with in vitro chemical transformation and neoplastic transformation. The inhibition of transformation from chemical treatment was not a result of cell killing in that sub-toxic concentrations were used.

Table 2. Correlation between inhibition of virus transformation, in vitro chemical transformation and neoplastic transformation.

Chemical Group	In Vitro Chemical Transformation[1]	Neoplastic Transformation[1]	Inhibition of ST-FeSV Transformation
I. Naphthylamines			
1. ANA	Yes	Yes	Yes
2. PANA	Yes	Yes	Yes
3. PBNA	Yes	Yes	Yes
II. Polycyclic Hydrocarbons			
1. BAP[2]	Yes	Yes	Yes
2. Pyrene	No	No	No
III. Hydrazines			
1. HZ	Yes	Yes	Yes[3]
2. MMH	No	No	Yes[3]
3. UDMH	Yes	Yes	Yes[3]
4. SDMH	No	No	Yes[3]

[1] Courtesy Dr. George Milo
[2] BAP - Benzo(a)pyrene
[3] Significant enhancement - 2 hrs. pre-infection

Enhanced virus transformation by HZ, MMH, and SDMH observed when cells were exposed 2 hrs. pre-infection, may be related to cell growth stimulation shown by these chemicals in dose survival studies (data not shown). Similar findings of cell stimulation have been observed with murine and feline lymphocyte cultures (7). The major effect on virus transformation (inhibition) occurred with all test chemicals when virus infected cells were exposed to the appropriate concentrations. The temporal relationship of chemical treatment to virus infection appears more critical with the hydrazines than with the naphthylamines in that maximum inhibition occurred when virus infected cells were exposed to the hydrazines 2 hrs. post-infection, whereas this inhibitory effect was observed at all 6 time periods with PANA and PBNA and at 3 time periods with ANA.

Cytotoxicity results showed no significant difference in shale oil or petroleum derived fuels. Co-carcinogenic effects with ST-FeSV are being evaluated. HZ, MMH, UDMH, SDMH, and PANA have shown mutagenic, teratogenic, or carcinogenic properties depending upon the assay used (7-11). Thus, these chemicals interact with host cell transcription or translational processes. In previous studies, we concluded the inhibitory (anti-carcinogenic) effect of benzo(a)pyrene, aflatoxin B1 or N-acetoxy-2 fluorenyl acetamide on virus transformation was not due to decreased cellular proliferation or virus synthesis (1,2). Further, the anti-carcinogenic effect was abrogated when cells were exposed >24 hrs. post-infection. The temporal relationship between infection and treatment suggested chemical interference with FeSV proviral synthesis or integration into host cell DNA.

Contrasting results have been reported on the interaction of chemical carcinogens and oncogenic RNA viruses. For example, in vivo studies showed either an anti-carcinogenic (12,13), co-carcinogenic (14,15) or no effect (16,17) on transformation depending on the virus or chemical used in the experiments. Whereas in vitro studies with rat or mouse cells showed synergism (18-20).

We previously reported anti-carcinogenic activity with three known carcinogens on FeSV transformation of human cells in vito. A recent report by Rhim and Arnstein (21) described anti-carcinogenic activity of an oncogenic murine virus on chemical-induced transformation of canine cells.

Thus, the mechanism(s) of chemical, virus, or co-chemical-virus transformation remain unknown and further studies are warranted to evaluate these interactions.

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Chapter 5

Factors Affecting Feline Retrovirus Infectivity and Oncogenicity

Introduction

Adult cats which are normally resistant to challenge with the laboratory strain of feline leukemia virus (FeLV) can be made susceptible to FeLV infection and disease if exposed to certain chemicals. Similarly, productive infection or transformation by feline sarcoma virus (FeSV) or FeLV can be induced in normally resistant cultured cell lines by treatment with various chemicals or physical agents. The objectives of this chapter are to: 1) provide a brief background of chemical-viral cocarcinogenesis; 2) review the specific experiments involving the manipulation of FeLV-SV infectivity and oncogenicity by various means; and 3) discuss possible mechanisms of these changes in susceptibility to FeLV-SV in light of the reported experiments.

Background

Chemical alteration of viral infectivity or oncogenicity has been studied since the turn of the century. Many chemical carcinogens are known to permit or enhance viral-induced neoplasia or transformation both in vivo and in cell culture systems (1). For example, in mice, urethan in conjunction with murine leukemia virus (MuLV) caused a much higher incidence of leukemia (13-31%) than MuLV alone (0-2%), or urethan alone (4%) (2). Chieco-Bianchi et al. (3) showed similar synergistic effects with urethan and MuLV in their experiments.

Another example of in vivo chemical-viral cocarcinogenesis was demonstrated by Andrewes et al. (4) and Alhström and Andrewes (5), using rabbit fibroma virus and benzo-(a)-pyrene or tar. Application of the chemicals with the virus caused more numerous, larger and more slowly regressing fibromas than the virus or chemical alone.

Rous and Friedewald (6) showed similar results in rabbits treated with Shope papilloma virus followed by topical treatment with tar or 3-methylcholanthrene (3-MCA), resulting in the appearance of squamous cell carcinomas. These tumors did not develop after treatment with virus or chemical alone.

Similar cocarcinogenic effects have been found in certain cell culture lines treated with various physical or chemical agents and oncogenic viruses, particularly DNA viruses. Data presented by Casto and DiPaolo (1) using a Simian adenovirus and Blakeslee et al. (7) using SV40 virus showed that cells treated with virus and with ultraviolet light, various classes of chemical carcinogens, or DNA base analogues showed an increased susceptibility to viral transformation. It has been postulated that certain classes of carcinogens damage cellular DNA, and enhanced transformation is the result of viral DNA being present during host cell DNA repair synthesis (3). Alternatively, viral DNA may be incorporated into cellular DNA at sites of unrepaired lesions during scheduled DNA synthesis (9). The insertion of viral DNA in unrepaired sites could occur in cells defective in or with reduced repair capabilities.

These observations suggest that at least certain chemical carcinogens as well as radiation enhance viral transformation by increasing the number of sites for integration of viral genetic material into cell DNA.

The above observations with a nonreplicating DNA virus system have not been as clearly substantiated for replicative RNA tumor virus systems. Nonetheless, others (10) have demonstrated that murine leukemia virus infected rat and mouse cells undergo transformation following additon of carcinogens such as 3-MCA, benzo-(a)-pyrene (B(a)P) and diethylnitrosamine (DENA). These studies suggest that chemical carcinogens activate viral coded oncogenic information which may be inherent in the cells, but which require the helper functions of the leukemia virus for expression. However, the

treatment of rat embryo cultures with 3MCA one to three weeks prior to additon of Rauscher Leukemia Virus (RLV) did not lead to transformation of these cells, whereas 3MCA treatment up to 3 weeks after RLV infection yielded transformed colonies within 7 to 10 passages. These data indicate that changes induced in the cell by certain chemical carcinogens were of a transient nature, and that the chemical treatment apparently did not permanently activate some endogenous agent which later participated in the process of virus transformation. The possibility of transient viral gene activation (derepression followed by repression) cannot be excluded.

In Vivo Enhancement of FeLV Oncogenicity by Methylnitrosourea: Occurrence and Possible Mechanism.

Adult specific-pathogen-free (SPF) cats are normally resistant to challenge with laboratory strains of FeLV, developing high feline oncornavirus-associated cell membrane antigen (FOCMA) and FeLV neutralizing antibodies, and transient or no viremia. Methylnitrosourea (MNU), a potent resorptive carcinogen of the nitrosamide family, was found to abolish this age-related resistance to FeLV when given in subcarcinogenic doses. In an initial experiment, 6 of 9 young adult cats treated with a single dose of 15 or 20 mg/kg MNU intravenously and inoculated with FeLV intraperitoneally (i.p.) became persistently viremic, while only 1 of 12 cats of the same age treated with FeLV alone became persistently viremic (11). Subsequent experiments showed that FOCMA antibody titers of FeLV + MNU treated cats were markedly lower than FeLV-treated cats (Table 1). It was further shown that the route of FeLV inoculation affected FeLV susceptibility in that the MNU-treated cats inoculated oronasally with FeLV showed a much lower incidence of viremia (1 of 5) compared to i.p. inoculation (6 of 9). However, MNU treatment of oronasally challenged cats did suppress FOCMA antibody titer (0.8 ± 0.5) compared to untreated oronasally challenged cats (4.0 ± 0.53).

A third experiment showed that MNU treatment did not alter pre-existing immunity to FeLV. Preimmune, MNU-treated cats were resistant to FeLV challenge, and developed a normal anamnestic antibody response to FOCMA. The results of all these experiments are summarized in Table 2.

Chemical carcinogens other than MNU have not been tested for their ability to enhance cats' susceptibility to FeLV. Noncarcinogenic agents which alter FeLV susceptibility, such as corticosteroids and silica, are discussed in Chapter 4.

Immunosuppression Induced by MNU

One possible mechanism to explain chemical-viral cocarcinogenesis, in addition to those previously mentioned, includes chemically-induced general immunosuppression. An immunosuppressed host would be unable to respond adequately to viral antigens and/or viral-induced tumor antigens on transformed cells, hence, neoplasia could occur more readily. Many chemical carcinogens have been proven to be immunosuppressive (12) either because of systemic toxic effects or specific suppression of the reticulo-endothelial system.

The effects of MNU on the feline immune system were investigated and, indeed, MNU proved to be highly immunosuppressive (13). Cats were given a single dose of 15 mg/kg intravenously. Cutaneous allograft retention time was markedly prolonged, from an average of 16 days for the control group to an average of 74 days for the MNU-treated group (Fig. 1, Table 3). Lymphocyte blast transformation (LBT) response of peripheral mononuclear cells (PMC) to both antigen and mitogens was markedly suppressed for up to three months following MNU injection. The response to both pokeweed mitogen and concanavalin A was suppressed by up to 50-fold compared to the average pretreatment control values (Fig. 2 and Fig. 3). Similarly, the LBT response to specific antigen of cats treated with MNU and simultaneously immunized with keyhole

limpet hemocyanin was delayed and significantly diminished compared to untreated control cats (Fig. 4).

MNU was further found to have a direct suppressive effect on the cells responsible for the LBT response (T-lymphocytes and/or macrophages) (14). When PMC from normal cats were incubated briefly with MNU, then washed and stimulated with con A in the LBT test, a definitive dose-related suppression occurred (Fig. 5). As little as 10 μg/ml of MNU induced a statistically significant suppression of the LBT response. This effect was not due to cytotoxicity in that the highest concentration of MNU tested (100 μg/ml) did not significantly reduce lymphocyte viability.

A related carcinogen, N-Methyl N-nitrosoguanidine (MNNG), also suppressed the LBT response of normal feline lymphocytes to con A after brief in vitro incubation (Fig. 6).

Factors Affecting In Vitro FeSV Infection

1. Chemical Carcinogens. The interactions of FeSV/LV with chemical and physical carcinogens and other exogenous factors affecting infectivity and oncogenicity have not been as extensively studied as the murine retrovirus system. We have used Snyder-Theilen Feline Sarcoma Virus (ST-FeSV) in a human cell line to investigate these factors.

The susceptibility of human cells to FeSV and FeLV infection has been well established (15-19). In vitro infection of normal human skin fibroblasts with ST-FeSV demonstrated "single hit" kinetics and produced foci of cells with altered morphology (Fig. 7). Initially, quantitive enhancement of ST-FeSV focus formation and proliferation of transformed cells was demonstrated with select hormones (20) (to be discussed later in Chapter). We extended these studies to include the effect of other chemicals with known carcinogenic activity on ST-FeSV focus formation. Carcinogens representative of known chemical classes of compounds, benzo (a) pyrene (BP), aflatoxin B1 (AFB), and

N acetoxy-2-fluorenyl acetamide (A-AAF) were investigated for their action on a quantitative virus directed focus forming assay (21).

As shown in Figs. 8 to 9, cells treated with sub-toxic doses of carcinogen resulted in significant inhibition of ST-FeSV directed transformation. Treatment with sub-toxic doses of the non-carcinogenic polycyclic hydrocarbon, pyrene, did not significantly reduce ST-FeSV focus formation, suggesting a possible relationship between carcinogenic potential and foci inhibition.

2. Hormones. Steroid hormones have been reported to enhance or inhibit viral transformation in vitro (7,20), modulate oncogenic DNA virus expression in nonpermissive cells (22) and to inhibit excision DNA repair with increased virus transformation in estrogen and chemical carcinogen-treated SV40 virus-infected human cells (7). Several reports have documented the stimulation of murine retrovirus synthesis in cell cultures exposed to optimal concentrations of glucogenic adrenocorticosterol hormones (23,24). More recently, Varnier and Levey (25) provided evidence that for xenotropic and the FMR strains of ecotropic endogenous murine leukemia viruses, replication is enhanced by dexamethasone (DXM). Schaller et al. (20) described quantitative and qualitative enhancement of ST-FeSV transformation in human neonatal foreskin cells when the DXM was added to infected cell cultures 24 hours post-infection. Additional hormones evaluated and shown to enhance focus formation were hydrocortisone, cortisol acetate and prednisone. No effect was detected with 17^{B} estradiol, progesterone, or methyl testosterone.

Blakeslee et al. (26) in further studies with DXM showed that non-DXM treated ST-FeSV infected human cells underwent morphological transformation (focus formation). However, little or no infectious virus was demonstrable in supernatant fluids from these cultures when added to the same strain of uninfected fibroblasts. The addition of 1.0 μg/ml of DXM 24 hours post-infection resulted in significant increases

(56X to 100X) in infectious FeSV (Table 4). Reverse transcriptase activity was likewise increased, ranging from a 9-fold increase to a 12-fold increase. Feline group-specific antigens (GSA) and FOCMA were detected in both DXM treated and nontreated FeSV infected cells. Uninfected cells, and cells treated with 0.2% acetone and/or DXM were negative (data not shown).

3. Asbestos. Asbestos is the commercial name for a group of naturally occurring, highly fibrous silicate minerals that readily separate into long, thin, strong fibers of sufficient flexibility to be woven. Industrial uses include cement, floor tiles, paper products, paint and caulking, brake linings, and cement-asbestos pipes.

Epidemiologic studies have shown occupational exposure can lead to increased risk of asbestosis, bronchogenic carcinoma, pleural mesothelioma and peritoneal mesothelioma. Ingestion or inhalation results in direct contact with epithelial cells lining the buccal cavity, esophagus, stomach and intestines.

As a continuation of studies in which FeSV transformation of human cells was used to develop an assay for determining potential carcinogens by their predictable effect on virus-induced transformation, 3 types of asbestos were used: Amosite, Chrysotile, and Crocidolite. Some properties are listed below:

Table 5. Characteristics of Asbestos Fibers.[1]

Characteristics	Chrysotile	Crocidolite	Amosite
Base Composition	Hydrate magnesium silicate	Hydrated silicate of iron and sodium	Hydrated silicate of iron and magnesium
Texture of fiber	Silky; soft	Harsh	Coarse
Flexibility and Spinning Properties	Excellent	Fair	Poor
Major Properties	Flexible heat resistant	Flexible heat resistant	Brittle

[1] Modified from Harrington, Allison and Badami

The results of experiments in which cells were infected with FeSV and treated either prior to or subsequent to infection with nontoxic concentrations of the 3 types of asbestos are shown in Figs. 11 to 13. Chrysotile- and Crocidolite-treated cells enhanced FeSV transformation (Figs. 11 and 12), whereas Amosite significantly inhibited transformation (Fig. 13).

As shown in Table 6, when virus was incubated with asbestos, then centrifuged to remove asbestos and the supernatant used to infect cells, a 60% reduction in virus transformation with Amosite was observed, suggesting adsorption of the virus to the Amosite. No such effect was noted with Chrysotile or Crocidolite. Concomitant treatment of cells with virus and asbestos resulted in significant enhancement of transformation (1.9 to 2.3 fold increase) with all 3 types (Table 7).

Relationship of Efficiency of DNA Repair, Age and Susceptibility to FeLV Infection

Various physical and chemical environmental agents have been shown to damage cellular DNA in vivo. Correctly repaired, the damage has little effect on the biological function of the system. Unrepaired damage, however, results in changes in physiological processes such as growth, transcription, mutation and induction of transformation. Thus, the more effective a cell is in the repair of genetic damage, the less sensitive it is to possible deleterious effects of environmental agents.

The efficiency of DNA repair can be measured by inducing DNA damage with ultraviolet light or chemicals, then measuring the rate of incorporation of tritiated DNA precursors into the repaired region. Using these techniques, less efficient DNA repair has been associated with the aging process and increased susceptibility to cancer.

Hart and Setlow (27) have related the expectant life (aging) of various mammalian species to the efficiency of DNA excision repair (one of three forms of DNA repair). In these studies, the initial rate of maximum incorporation of (^{3}H) dThd increased with life

span. Of the species tested, the amount of unscheduled DNA synthesis (DNA repair) was greatest in man, >elephant, >cow, >hamster, >rat, >mouse, >shrew. The extent of excision repair implied that cells proficient in such repair removed more damaged DNA than cells deficient in repair. Hence, over a given period of time, a mouse might accumulate in its DNA more damage per unit length than would a man, accounting for the differences in life span.

Increased susceptibility to virus-induced cellular transformation has been associated with the capacity for DNA repair. Blakeslee and Milo, in their studies with SV40 virus and chemical carcinogens, found that only those carcinogens which induced DNA damage enhanced SV40 transformation (28). Further, it was shown that hormonal inhibition of unscheduled DNA repair resulted in significant enhancement of virus transformation after DNA damage by a radiomimetic chemical (7).

Thus, the efficiency of DNA repair may have a direct relationship to the susceptibility of cells to viral integration and viral induced transformation.

Studies were undertaken to determine if a relationship existed between feline retrovirus induced disease and efficiency of DNA repair in feline fibroblast cells grown from surgical biopsies from different aged cats. Cellular DNA was damaged by one hundred ergs/mm^2 UV, scheduled DNA synthesis inhibited by arginine-free medium and hydroxyurea, and as a measurement of unscheduled DNA synthesis, ^{3}H-thymidine incorporation over a 24-hour time period was used to measure the extent and rate of repair.

The results of this study are shown in Fig. 14. The initial rate of repair up to 4 hours was similar regardless of the cat's age at the time of biopsy (Panels A & B). However, with increasing age, a decrease in the extent of repair was seen. Panel C depicts the results of repair in 3 littermates in order to determine variation within a

group of similar genetic make-up. As shown, no such variability was detected; the rate and extent of repair was similar with cells from the 3 kittens.

Discussion

Alteration of animal or cellular susceptibility to FeLV/SV infection by chemical or physical agents is a well recognized phenomenon. Several possible mechanisms are suggested by the preceeding experiments. Carcinogens, for example, may cause a generalized immunosuppression as shown in our experiments with MNU and cats, and this immunosuppression may account for the increased susceptibility to FeLV infection. In an immunocompetent animal exposed to FeLV, target cells may be continually infected and transformed, but the foreign viral-coded antigens such as FOCMA, which are expressed on the cell surface, elicit an immune response and the transformed cells are destroyed before they have a chance to become established. In an immunosuppressed animal, however, the transformed cells are less efficiently eliminated and thus able to establish a large population, resulting in neoplasia. To support this theory, most oncogens, when used in oncogenic doses, interfere with normal immunologic reactions (12).

On a cellular level, it appears that the cells involved in the immune response (lymphocytes and/or macrophages) are directly suppressed by carcinogens such as MNU, as evidenced by the decreased LBT response to con A after incubation with MNU. Other carcinogens which induce a dose-related suppression of the LBT response after or during in vitro incubation with lymphocytes include MNNG, hydrazine, and 1,1-dimethylhydrazine (Tarr, unpublished data).

Exposure to immunosuppressive compounds other than carcinogens, such as dexamethasone or silica, will also increase cats' susceptibility to feline retrovirus infection, as discussed in Chapter .

The use of in vitro cell culture systems allows the study of cell, virus, and chemical interactions at a molecular level, and may help elucidate the mechanisms of cocarcinogenesis. The experiments described in this chapter suggest several possible mechanisms.

Protein synthesis is one cellular function which may be affected by chemicals, resulting in an alteration of the normal host cell-virus relationship. Other investigators (29-31) have reported that carcinogens such as AFB1, A-AAF and B(a)P interfere with host cell translational mechanisms. Thus, chemical carcinogens may inhibit feline retrovirus-induced transformation by interfering with provirus synthesis or integration of proviral DNA into cellular DNA. In support of this concept, it is known that for avian and murine retroviruses, synthesis and transport of proviral DNA from the cytoplasm to the nucleus occurs between 6 and 24 hours post infection (32,33). An experiment described by Blakeslee and Milo (21) showed that B(a)P or AFB1 treatment 24 hours post-infection had little or no effect on transformation of FeSV-infected cells. This temporal relationship suggests that interference with proviral synthesis or integration has occurred.

Chemicals may also affect the normal interaction between virus and cell by altering viral gene expression. Wu et al. (34) described retrovirus infected cells as having 3 phenotypic categories with respect to virus gene expression: 1) virus producer cells, 2) non-producer cells with partial gene expression (any virus component) and 3) cells with no detectable viral gene products. Our experiments showed that BP or AFB2-treated and retrovirus-infected cells as well as cells infected with retrovirus alone produced no infectious virus, but demonstrated comparable levels of RT activity, and expressed FOCMA and GSA. These data indicate that: 1) the human skin fibroblasts used in these studies were classified as non-producer cells with partial viral gene

expression, i.e. transformation, RT activity, GSA and FOCMA, 2) virus synthesis was not affected by the chemical carcinogen treatment, and 3) the inhibitory effect on virus transformation was chemically mediated while virus synthesis was host-cell mediated.

In contrast to B(a)P and AFB, DXM stimulated expression of all viral gene products, although not to the same degree. The RT activity was stimulated 9- to 12-fold, whereas release of infectious virus was stimulated 56- to 100-fold. This can be explained if there is partial gene expression (i.e. RT activity) without DXM treatment, which is indicated by the relatively high ^{3}H TMP incorporation in the RT assay using fluids from non-DXM treated, virus infected cells. Ahmed et al. (35) reported similar findings in that DXM-treated, Mason-Pfizer retrovirus-infected primate cells contained an 8-fold increase in RT activity and a 10-fold increase in infectious virus titer.

The increased virus synthesis in DXM treated cells is not the result of hormone-induced proliferations of cells in that previous studies by Schaller et al. (20) showed that 1.0 μg/ml DXM inhibited cell proliferation with increased yields of virus. The stimulatory-activity of DXM on retrovirus gene expression has been extensively studied and appears to be at a post-transcriptional step in virus replication (24,26,34,35,36).

The interaction of asbestos and feline retrovirus reveals yet another possible mechanism of chemical-viral alteration of cell activity. Electron micrographs show that all 3 fiber types are ingested by the fibroblast cells used in this study (37), and X-ray diffraction analyses of the treated cells revealed a loss of Mg^{++} and Fe^{+++} from the fibers within the cytoplasmic matrices. The order of loss was Amosite >Crocidolite >Chrysotile. Thus, leaching of the cations from the ingested asbestos fibers appears related to the effect on FeSV transformation in that cells treated with Amosite resulted in significant inhibition of transformation, whereas cells treated with Chrysotile and Crocidolite resulted in enhancement of transformation. The increased levels in Mg^{++}

may provide an increased cellular pool of cations required for viral replication and transformation (Crocidolite and Chrysotile) or, in the case of Amosite, a feed-back mechanism inhibiting cellular metabolic functions such as viropexis.

Intrinsic DNA repair mechanisms may also be a factor which affects cellular susceptibility to viral infection or transformation. On an organism level, there is a well established age-related susceptibility in cats to FeLV infection (38), with very young animals being most susceptible, and developing increasing resistance such that they are nearly refractory to challenge by 8-12 weeks of age. Our data using cells from different aged cats suggest that susceptibility to FeLV may be inversely related to excision DNA repair. During the leukemia susceptible period of up to 8 weeks of age, the extent of excision repair peaks at approximately 5 weeks, and by 10 weeks of age, a 4-fold decrease in the extent of repair was noted. Thus, during the period of susceptibility to FeLV infection, maximum repair synthesis also occurs. Further studies are required to determine if, during repair, more sites are available for FeLV proviral integration and whether increased FeLV genome equivalents are detectable in these cells.

The use of in vitro systems to study the mechanism(s) involved in the complex interactions between retroviruses and chemical and physical carcinogens in the induction of neoplastic disease may eventually lead to understanding the process of transformation of a normal cell to a neoplastic cell. However, certainly other factors must be considered, including genetic makeup, influence of age, environmental factors, physiological factors, stress, hormonal levels, and the host's immune response in the susceptibility of the cat to FeLV and FeSV infection and disease.

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Table 1. Comparison of incidence of viremia and LSA disease and FOCMA antibody titer in cats inoculated intraperitoneally with FeLV and treated or not treated with MNU intravenously.

Cat No.	Rx	FeLV Viremia	Highest FOCMA of titer	LSA disease
597	FeLV	-	16	-
728		-	32	-
729		-	32	-
735		-	64	-
743		-	64	-
744		-	16	-
672	FeLV + MNU	+	4	+
676		+	4	+
701		+	4	+
703		-	64	-
706		+	4	+
707		+	4	+
718		+	4	+
734		-	128	-

Table 2. Proportion of Cats Developing Viremia, FOCMA Antibody, or LSA Disease Following Exposure to MNU and/or FeLV.

Treatment Groups	Mean Age at Exposure (mo)	No. of Cats Developing Persistent Viremia /Total No. Tested	No. of Cats Developing FOCMA Antibody /Total No. Tested	Geometric Mean Highest FOCMA Antibody Achieved	No. of Cats Developing LSA Disease /Total No. Tested
FeLV (IP)	6.2 ± 0.4	0/6 (0)[b]	6/6 (100)	20.0 ± 0.49[c]	0/6 (0)
FeLV (IP) + MNU	6.1 ± 1.0	6/8 (75)	1/8 (13)	1.3 ± 0.75	6/8 (75)
FeLV (O/N)	35.1 ± 20	0/8 (0)	6/8 (75)	4.0 ± 0.53	NA[d]
FeLV (O/N) + MNU	25.2 ± 15	1/5 (20)	1/5 (20)	0.8 ± 0.50	NA
FeLV (IP) ± MNU (Pre-immune)[a]	7.5 ± 3.1	0/12 (0)	12/12 (100)	45.0 ± 0.51	NA

a) All 12 cats in this group had been exposed previously to FeLV at 4 mo. of age and carried persistently moderate to high FOCMA titers (mean = 24 ± 0.39).
b) Number in brackets represents the percent of cats responding.
c) Geometric mean FOCMA antibody titer ± standard error of the mean.
d) NA = not applicable.

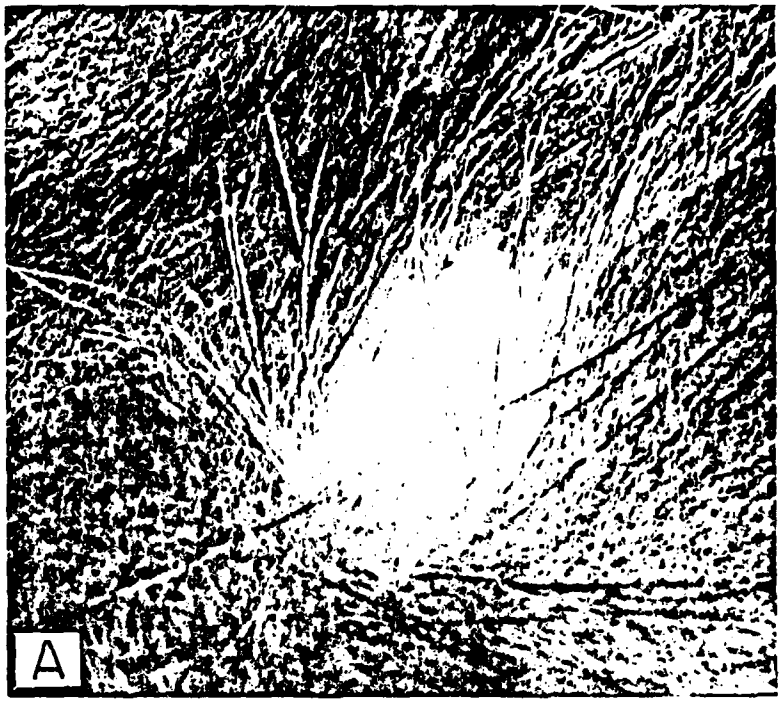
Table 3. Effects of MNU on Cutaneous Allograft Retention.

MNU Treated Cats		Untreated Control Cats	
Animal No.	Graft Rejection Time	Animal No.	Graft Rejection Time
756	84d	747	15d
800	84d	813	17d
802	45d	814	17d
803	84d	819	16d
mean	74.25	mean	16.25d

Figure 1. Cutaneous allografts in MNU-treated and control cats.

A) Cat number 800B, 63 days after MNU administration and skin graft. Note white hair growth from edges of non-pigmented grafted skin.

B) Cat number 819B, control, showing complete graft rejection with necrosis and scaling 17 days following the grafting procedure.


A

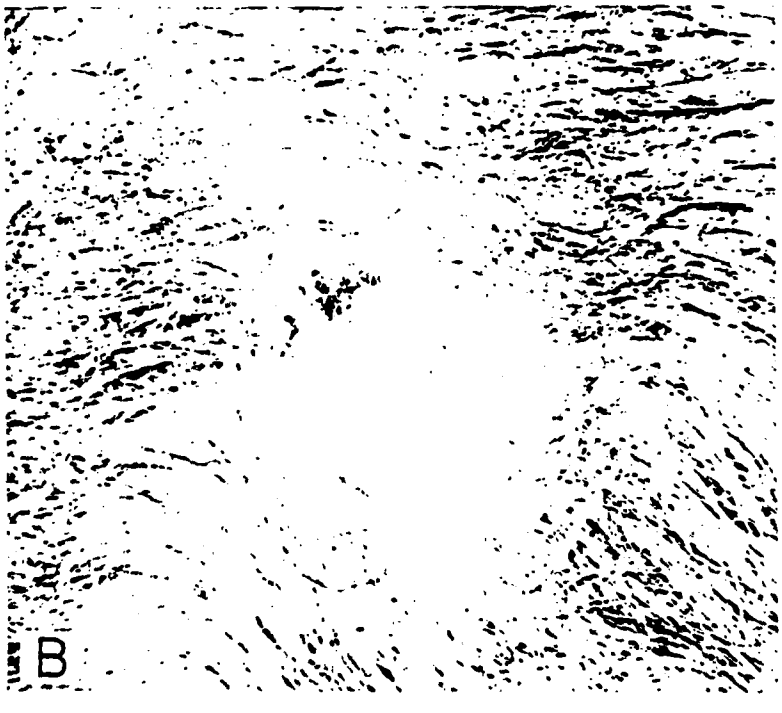

B

Figure 2. Lymphocyte blast transformation response of MNU-treated and control cats to con A. Vertical bars represent standard error of the mean (n = 4).

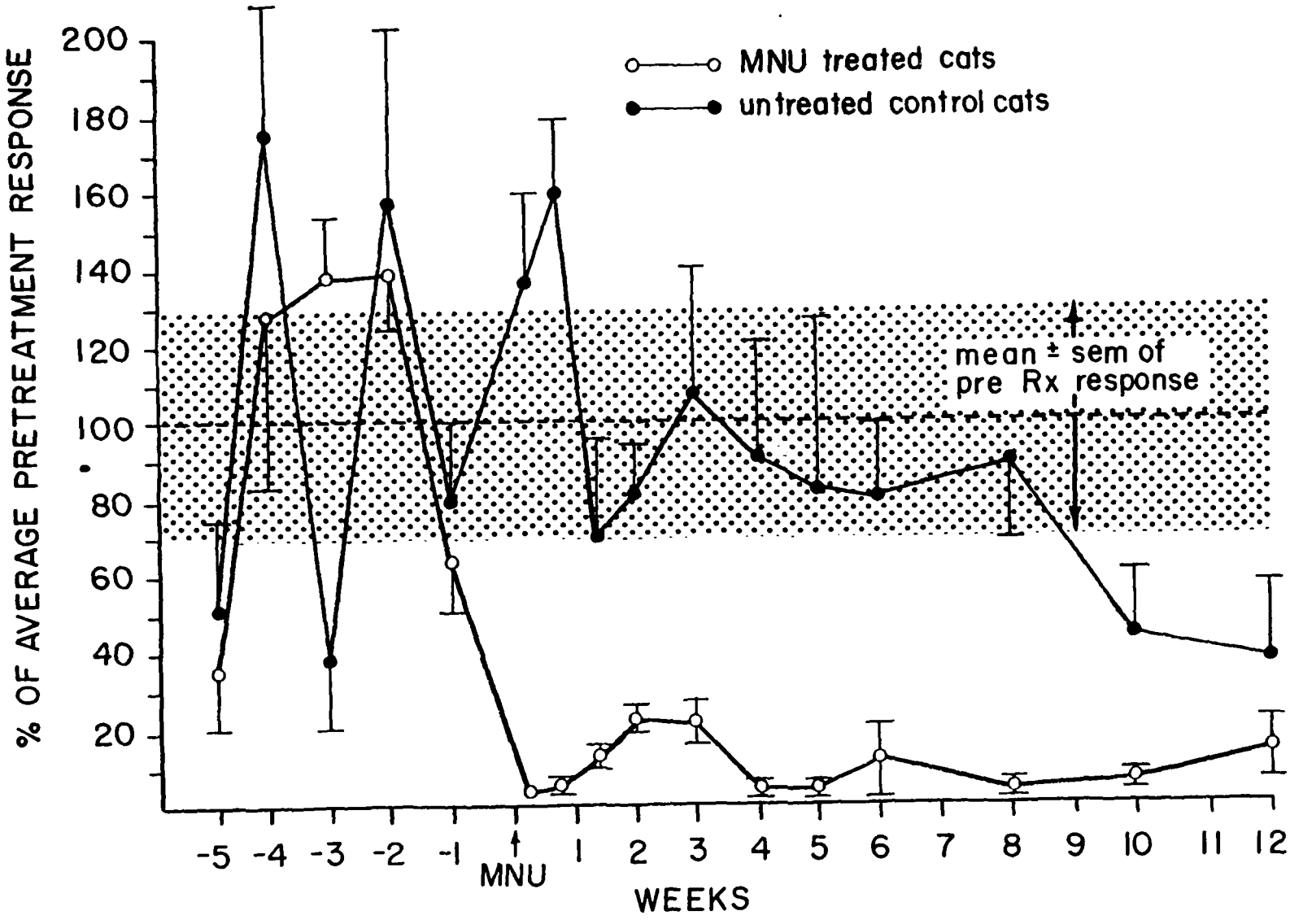

MNU treated cats
untreated control cats
mean ± sem of
pre Rx response
% OF AVERAGE PRETREATMENT RESPONSE
200
180
160
140
120
100
80
60
40
20
-5 -4 -3 -2 -1 MNU 1 2 3 4 5 6 7 8 9 10 11 12
WEEKS

Figure 3. Lymphocyte blast transformation response of MNU-treated and control cats to PWM. Vertical bars represent standard error of the mean (n = 4). Rx = treatment.

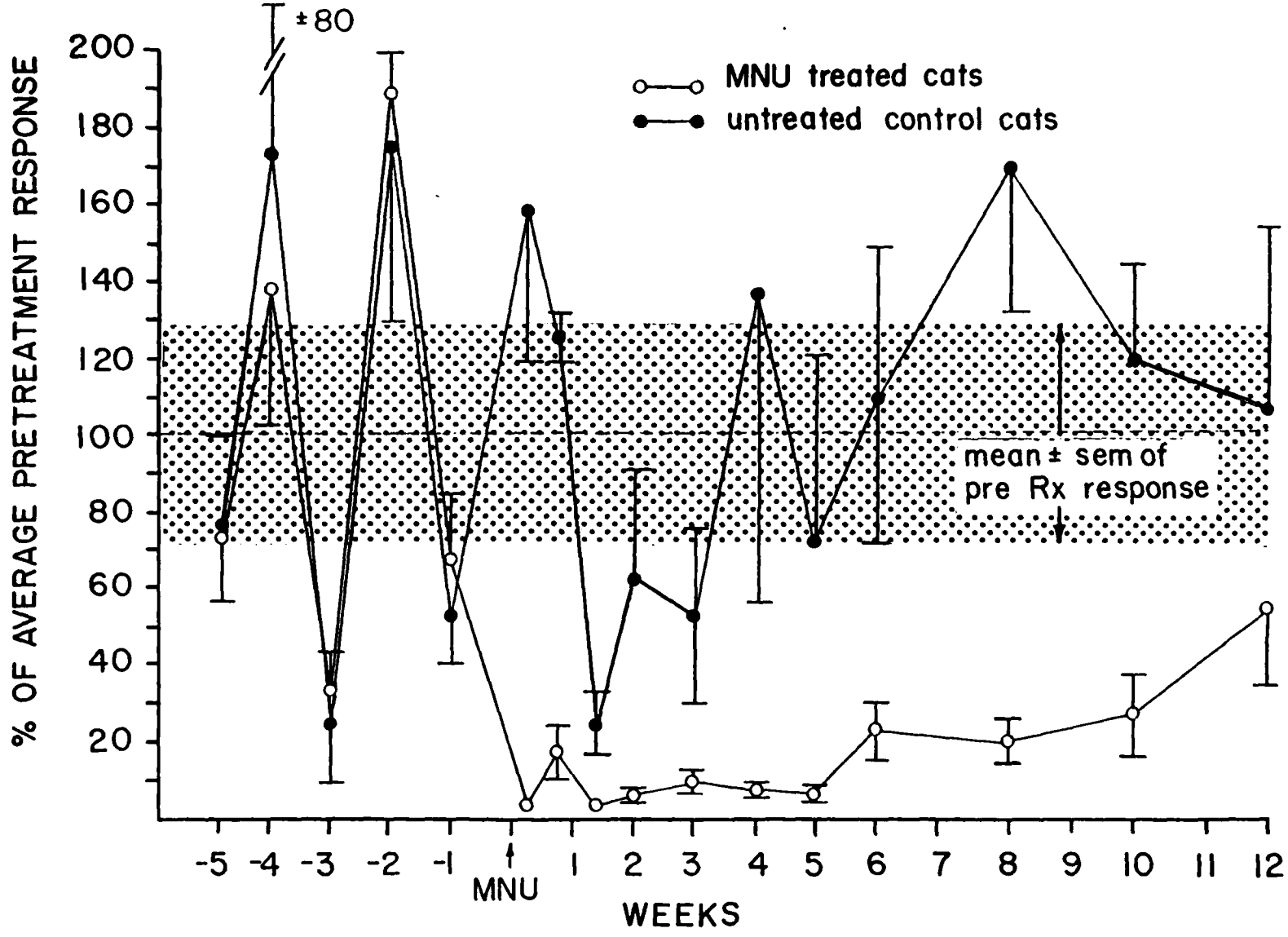

±80
200
180
160
140
120
100
80
60
40
20
% OF AVERAGE PRETREATMENT RESPONSE
MNU treated cats
untreated control cats
mean ± sem of
pre Rx response
-5 -4 -3 -2 -1
MNU
1 2 3 4 5 6 7 8 9 10 11 12
WEEKS

Figure 4. Lymphocyte blast transformation response of MNU-treated and control cats to KLH following KLH immunizaiton. Vertical bars represent standard error of the mean (n = 4).

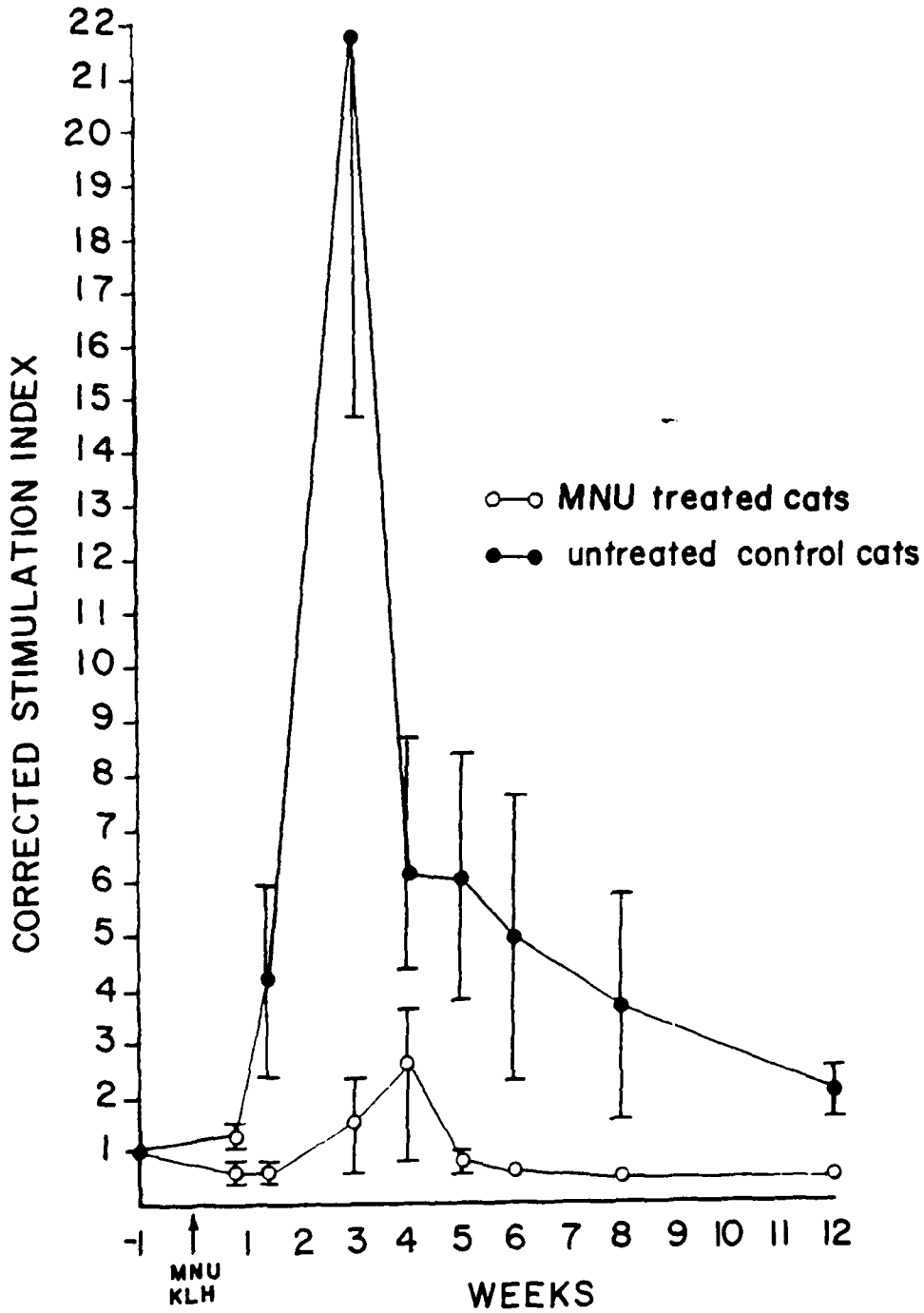

CORRECTED STIMULATION INDEX
22
21
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
MNU treated cats
untreated control cats
-1
MNU
KLH
1
2
3
4
5
6
7
8
9
10
11
12
WEEKS

Figure 5. Effects of initial MNU incubation of PMC on LBT response to con A. Points represent (CPM of MNU + con A cultures/CPM of control con A cultures) X 100. Vertical bars represent standard error of the mean (n = 10).

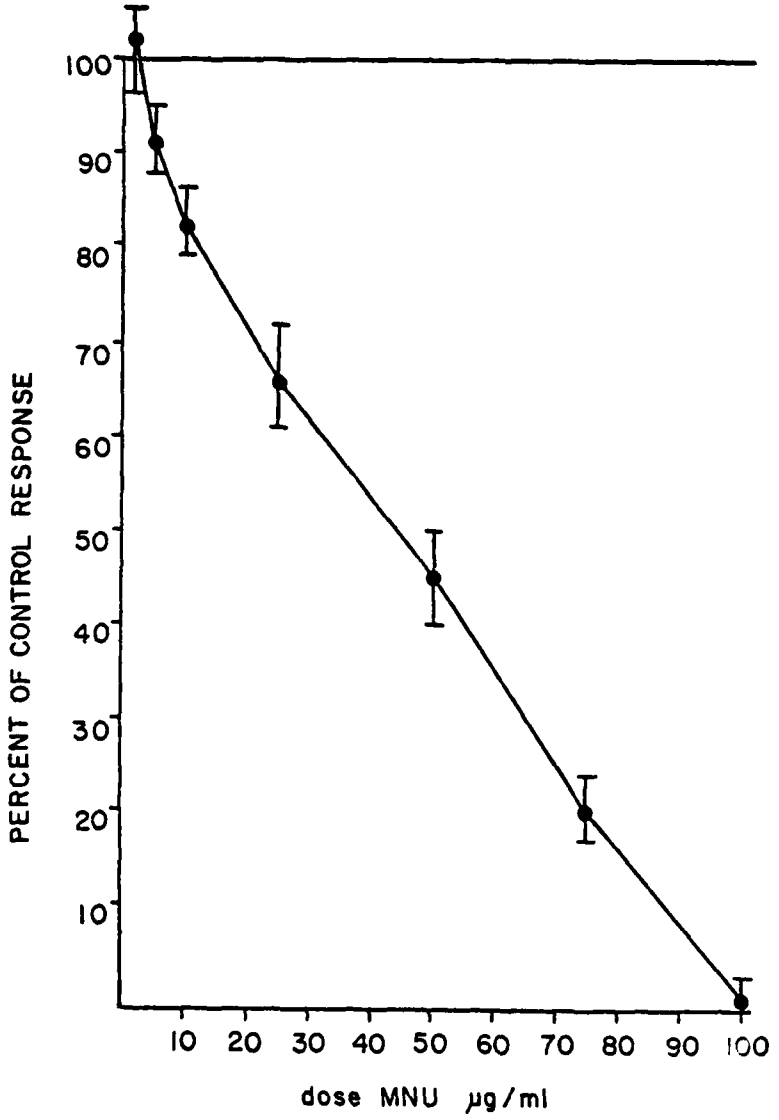

PERCENT OF CONTROL RESPONSE
100
90
80
70
60
50
40
30
20
10
10 20 30 40 50 60 70 80 90 100
dose MNU µg/ml

Figure 6. Effects of initial MNNG incubation of PMC on LBT response to con A. Points represent (CPM of MNNG + con A cultures/CPM of control con A cultures) X 100. Vertical bars represent standard error of the mean (n = 5).

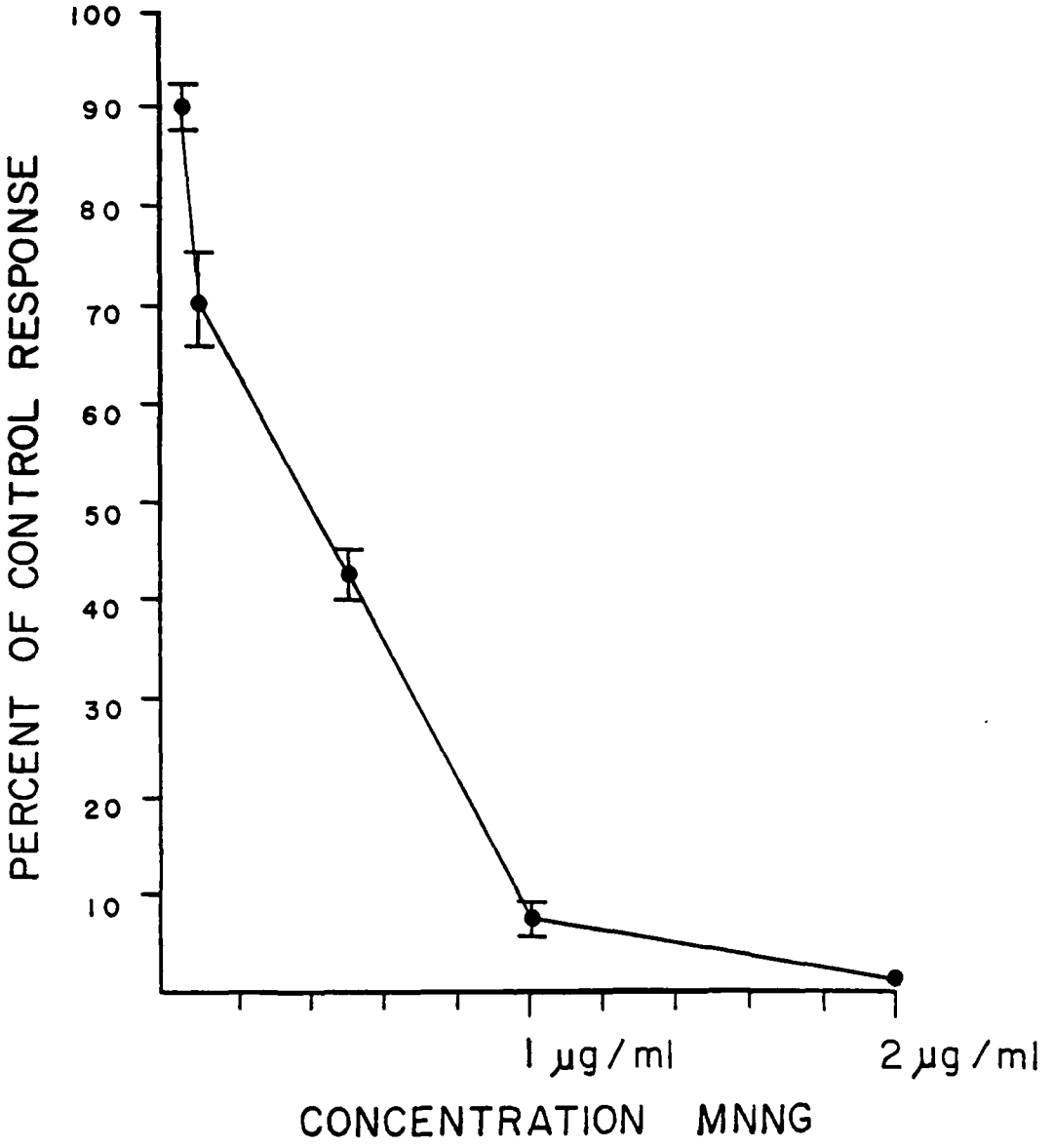

100
90
80
70
60
50
40
30
20
10
PERCENT OF CONTROL RESPONSE
1 µg / ml
2 µg / ml
CONCENTRATION MNNG

Figure 7. ST FeSV infected human skin fibroblasts. Twelve days post-infection (←—) foci of infected cells. Phase contrast X 56.

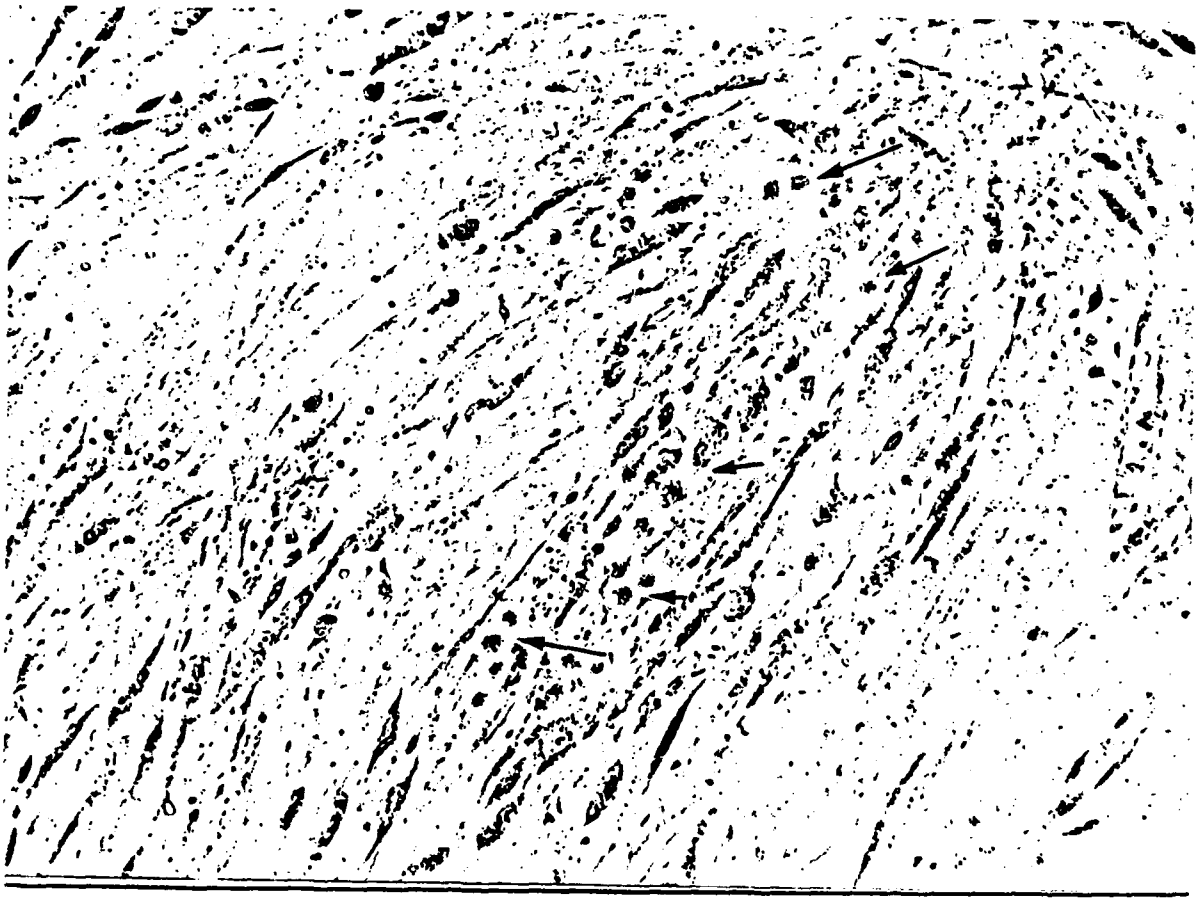


Figure 8. Inhibition of ST FeSV transformation by benzo (a) pyrene.

D550 cells (1×10^5) were plated in 35 mm diameter wells with 4 ml of growth medium and incubated 18 h. (—) designated cells treated with BP before virus infection. (+) designated cells treated after virus adsorptionfor 24 h with BP as described in Materials and Methods. Cultures were washed and fed with growth media at the end of each treatment period and at 6 days post-virus infection. The cells were subsequently fixed and stained 3-4 days later. Virus induced foci were counted in non-treated and chemically treated wells. Horizontal line at 0 represents virus infected controls. Percentage inhibition was determined by:

$$100 = \frac{\overline{\text{FFU chemically treated}}}{\overline{\text{FFU control}}}$$

Significance was determined by Student's t-test. Concentrations of BP used were: Δ, 15.0 μg/ml; o , 5.0 μg/ml; ▲ , 1.0 μg/ml; *, significant inhibition.

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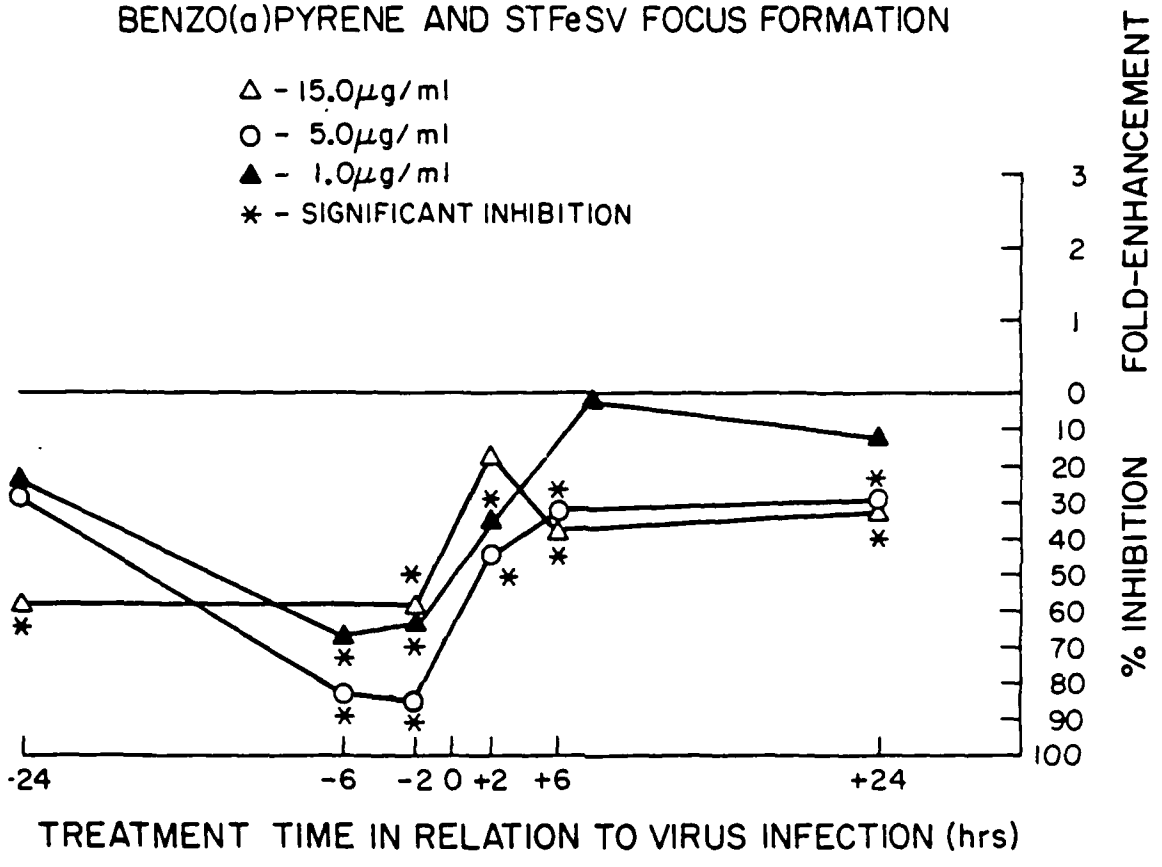
BENZO(a)PYRENE AND STFeSV FOCUS FORMATION
Δ - 15.0μg/ml
O - 5.0μg/ml
▲ - 1.0μg/ml
* - SIGNIFICANT INHIBITION
3
2
1
0
10
20
30
40
50
60
70
80
90
100
FOLD-ENHANCEMENT
% INHIBITION
-24
-6
-2
0
+2
+6
+24
TREATMENT TIME IN RELATION TO VIRUS INFECTION (hrs)

Figure 9. Inhibition of ST FeSV transformation by N-acetoly-2-fluorenyl Acetamide.

D550 cells (1×10^5) were plated in 35 mm diameter wells with 4 ml of growth medium and incubated 18 h. Cells were treated with A-AAF as described in Fig. 8. Data were plotted as described in Fig. 8. Concentrations of A-AAF used were: Δ, 1.0 μg/ml; o, 0.1 μg/ml; *, significant inhibition.

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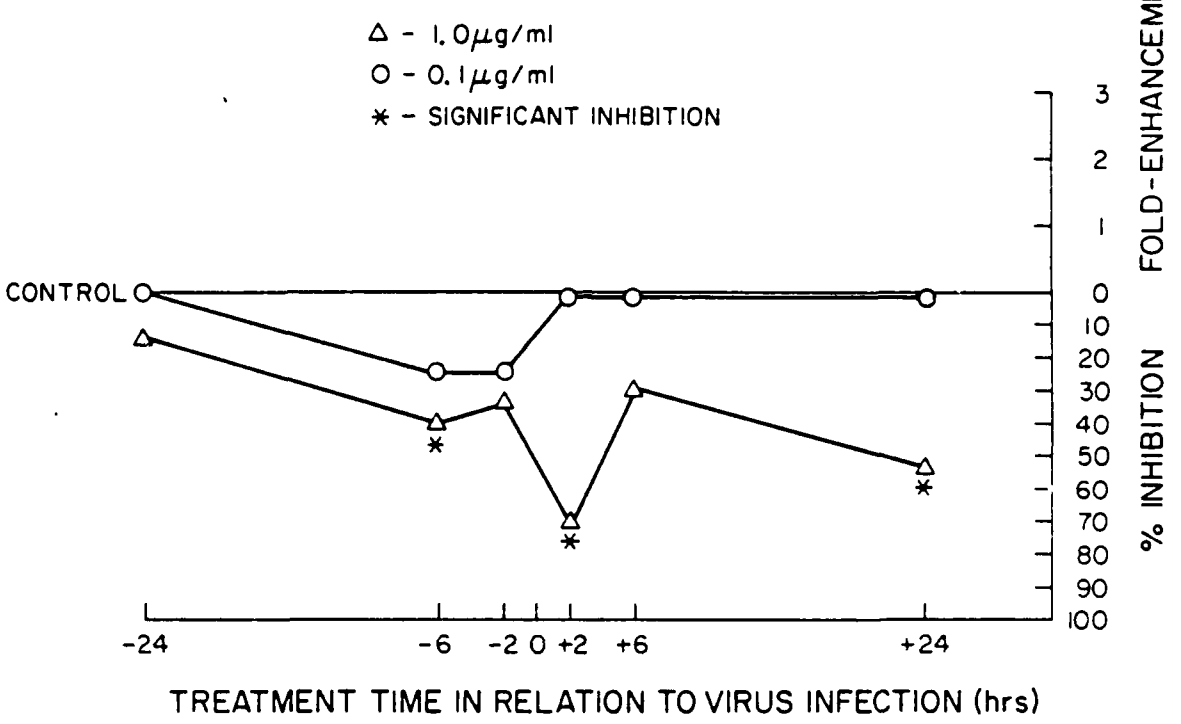
N-ACETOXY 2-FLUORENYL ACETAMIDE AND ST Fe SV FOCUS FORMATION



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MOLECULAR INTERACTIONS OF HIGH ENERGY FUELS AND JET FUELS WITH --ETC(U)
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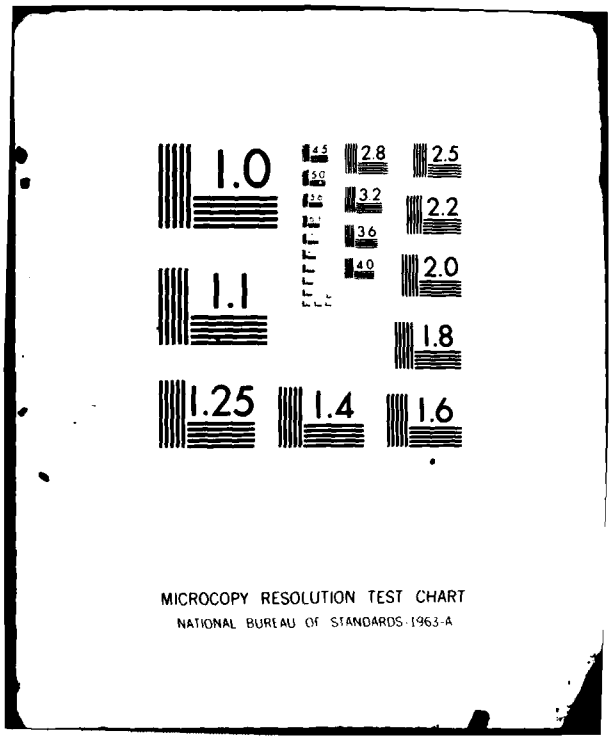

Figure 10. Inhibition of ST FeSV transformation by aflatoxin B1.

D550 cells (1×10^5) were plated in 35 mm diameter wells with 4 ml of growth medium and incubated 18 h. Cells were treated with AFB1 as described in Fig. 8. Percentage inhibition data was plotted as described in Fig. 8. Enhancement was determined by dividing $\overline{FFU}$ treated cells by $\overline{FFU}$ control cells. Significance determined by Student's t-test. Concentrations of AFB1 used were: Δ, 1.0 μg/ml; o, 0.1 μg/ml; *, significant inhibition.

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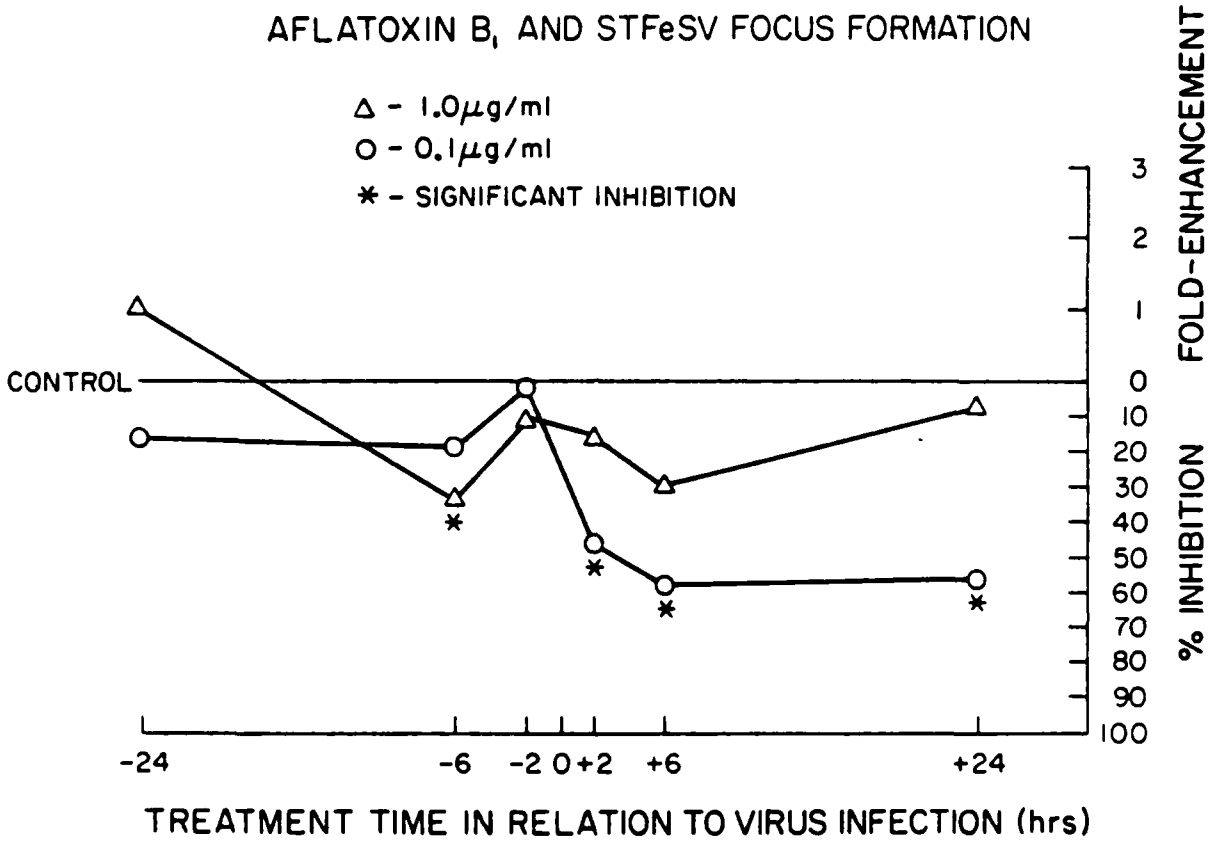

AFLATOXIN B1 AND STFeSV FOCUS FORMATION
Δ - 1.0μg/ml
O - 0.1μg/ml
* - SIGNIFICANT INHIBITION
CONTROL
3
2
1
0
10
20
30
40
50
60
70
80
90
100
% INHIBITION FOLD-ENHANCEMENT
-24
-6
-2 0 +2
+6
+24
TREATMENT TIME IN RELATION TO VIRUS INFECTION (hrs)

Legend Table 4:

Detroit 550 cells (1.5×10^6) were initially seeded into T-75 flasks and incubated 24 h. Medium was removed and cells infected as described in Materials and Methods. Ten days later, medium was replaced with 10 ml of growth medium and 24 h later, cells were harvested by either scraping or trypsinizing and processed as described below:

a) Cells removed with rubber policeman into the 10 ml of growth medium and q.s.'d to 20 ml with fresh growth medium and subjected to 1 cycle of freezing and thawing, and gross debris removed by 600 X g centrifugation. Two ml of clarified medium was used for infectivity assays and 18 ml used for reverse transcriptase assay.

b) Cells removed by trypsinization, centrifuged, growth medium discarded and cells resuspended in 20 ml fresh growth medium and further processed as described in (a).

c) Cells removed by trypsinization and processed as described in (a).

d) Ten ml of supernatant frominfected flasks was q.s.'d to 20 ml with fresh medium and subjected to 1 cycle of freezing and thawing and further processed as described in (a).

e) Procedures previously described ( ). Rickard FeLV twice banded in sucrose run as standard in RT assay: 52,075 cpm.

Figure 11. Interaction and effect of Crocidolite asbestos on ST FeSV transformation.

Cells (1×10^5) were plated in 35 mm diameter wells with 4 ml growth medium and incubated 18 h. (–) designated cells treated with Crocidolite Asbestos before virus infection; (+) designates cells treated after virus adsorption for 24 h with asbestos suspended in HBSS. Cultures were washed and fed with growth medium at the end of each treatment period and at 6 days post-virus infection. Cells were subsequently fixed and stained 3-4 days later. Virus induced foci were counted in non-treated and asbestos treated wells. Horizontal line at 0 represents virus-infected control.

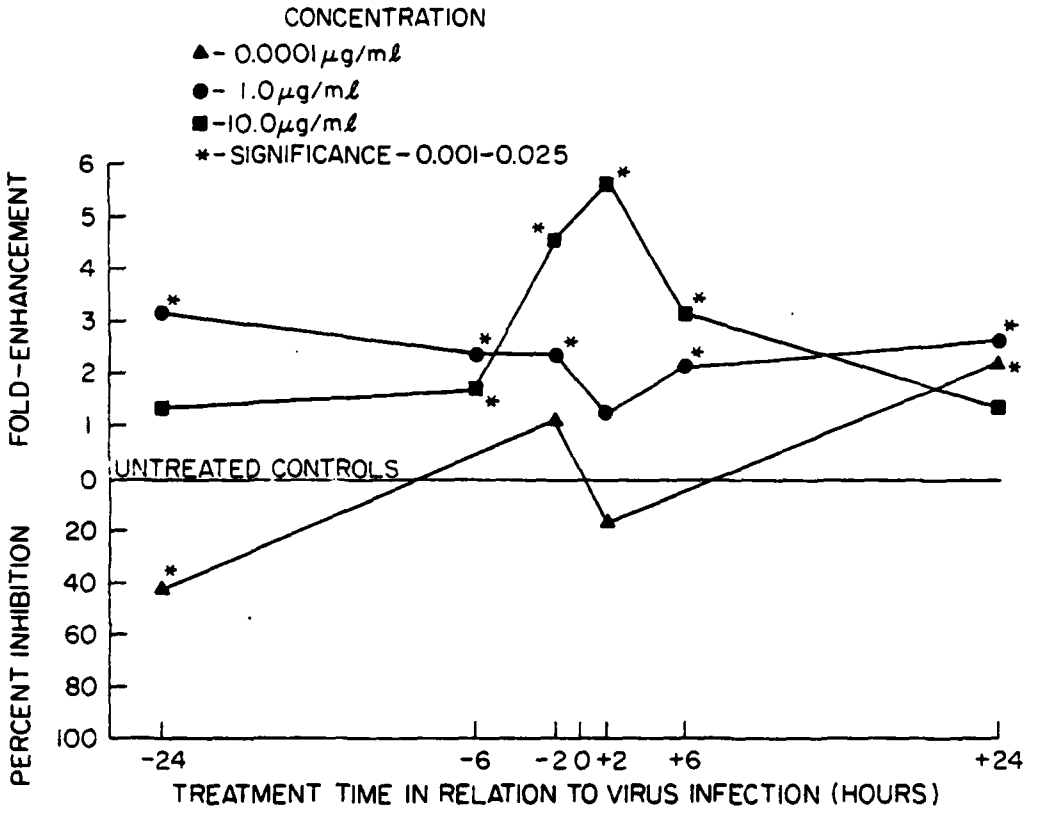
Percentage inhibition was determined by:

$$100 = \frac{\overline{\text{FFU}} \text{ asbestos treated}}{\overline{\text{FFU}} \text{ virus control}}$$

Fold enhancement determined by:

$$\frac{\overline{\text{FFU}} \text{ asbestos treated}}{\overline{\text{FFU}} \text{ virus control}}$$

Significance determined by Student's t-test (n = 24).


CONCENTRATION
▲- 0.0001 μg/mℓ
●- 1.0 μg/mℓ
■-10.0 μg/mℓ
*-SIGNIFICANCE-0.001-0.025
FOLD-ENHANCEMENT
PERCENT INHIBITION
6
5
4
3
2
1
0
20
40
60
80
100
UNTREATED CONTROLS
-24
-6
-2
0
+2
+6
+24
TREATMENT TIME IN RELATION TO VIRUS INFECTION (HOURS)