IN VITRO CHEMICAL CARCINOGENESIS AND CO-CARCINO-GENESIS IN HUMA--ETC(U)
SEP 80  G E MILO, J P BLAKESLEE
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AFOSR-TR-81-0273
IN VITRO CHEMICAL CARCINOGENESIS AND CO-CARCINOGENESIS IN HUMAN CELLS INITIATED BY HYDRAZINE AND POLYNUCLEAR COMPONENTS OF JET FUEL

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For the Period
July 1, 1977 - June 30, 1980

DEPARTMENT OF THE AIR FORCE
Air Force Office of Scientific Research
Bolling Air Force Base, D.C. 20332

Contract No. F49620-77-C-0110

September, 1980

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Examination of the carcinogenic activity of selected hydrazine, polynuclear hydrocarbons and/or napthylamines on human cells was evaluated on human foreskin cell populations in vitro. We also evaluated the interaction of these compounds with feline oncornavirus (ST-FeSV). To date we understand how this system can be used to evaluate the carcinogenic activity of the above suspected carcinogens. In Table 1, Segment 2, we have listed the chemicals evaluated in this system for their carcinogenic activity. Table 4 presents characteristics of the (continued)
Block 20 - Abstract (continued)

transformed cells in early, transitional and late stages of the carcinogenic process. The content to Table 2 Segment 2 summarizes the comparative aspects of the carcinogenic activity and cocarcinogenic activity of the chemicals. In Table 2 Segment 2 it was found that RDDP activity was absent in the carcinogen transformed cells. We can conclude from these data that the induction of the carcinogenic process is not a consequence of indogenous oncornavirus activation. These data presented to date illustrate that human cells can be used to evaluate carcinogens (Segment 1) and cocarcinogens (Segment 2) on human cells.
Final Report to
AIR FORCE OFFICE OF SCIENTIFIC RESEARCH
Directorate of Life Sciences
Bolling A.F.B., D.C. 20332

Title: IN VITRO CHEMICAL CARCINOGENESIS AND CO-CARCINOGENESIS
IN HUMAN CELLS INITIATED BY HYDRAZINE AND
POLYNUCLEAR COMPONENTS OF JET FUEL

Inclusive Dates of Report:
July 1, 1977 - June 30, 1980

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Technical Information Officer
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<td>IX</td>
<td>Support Data and 16 copies of each</td>
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</table>
1. a) Work Scope: - 1977

1. Examine the cytotoxic and carcinogenic potential of hydrazine and polynuclear hydrocarbons, components of jet and rocket fuel on human cell populations, in vitro.

2. Investigate damage to cellular DNA by studying DNA repair synthesis and alterations in cellular associated DNA directed DNA polymerase activities.

3. Investigate the post-translational responses to the cellular insults reflective of cytotoxic and carcinogenic responses.

4. Investigate, with oncogenic DNA and RNA viruses, whether the suspect carcinogens act synergistically with these viruses to enhance virus directed transformation of human cells.

5. Determine whether these chemicals damage cellular DNA, providing additional sites for virus integration.

The chemicals selected for investigation by Air Force representatives, Colonel V. Carter, Drs. K.C. Back, Berry and Ms. Marilyn George and The Ohio State University Research Foundation representatives Drs. George E. Milo and James Blakeslee were the following

a-0-napthylamine (-1-)
\( \beta \)-0-napthylamine (-2-)
\( \alpha \)-napthylamine' (-1-)
\( \beta \)-napthylamine (-2-)
monomethyl hydrazine
1,2-dimethyl hydrazine
hydrazine
symmetrical (1,1-) dimethyl hydrazine

We have also received some analogues of the arylamines that have been inserted into the regimen because they represent metabolites and or non-active analogues found in biological systems in response to treatment with the parent napthylamines.

Our interest in these agents is because, hydroxyl-arylamines have been shown to function as bladder carcinogens in dogs, (Kadilubar, et al., 1978). Apparently, the napthylamines are activated by activating enzymes at other sites in the animal, possibly the liver, and the hydroxyl derivatives glucuronated and transported to the bladder.

Therefore the following analogues were evaluated.

N-OH-1-Naphthylamine
N-OH-2-Naphthylamine
\( \alpha \)-NO\(_2\)-naphthylamine
\( \beta \)-NO\(_2\)-naphthylamine
4-aminobiphenyl
The system used in Segment I treated with these aforementioned compounds were fibroblasts isolated from human neonatal foreskins taken from the hospital at random. The system used for Segment II was a Detroit 550, a cell line developed from a single human foreskin. The viruses selected to be used in this study were SV-40 and FeSV.

The long-range goal of these studies will be to develop a human in vitro assay system that can be used to screen chemicals for potential carcinogenicity within a short period of time. Secondly, to understand how these chemicals act to induce carcinogenesis. Interrelated with this effort will be the use of viruses in the system to investigate the stages in the carcinogenesis process induced by the virus and to study the interaction between the human cells, transforming virus and suspect chemical carcinogens.

b) In 1977 our progress in Segment I.

1. Cytotoxicity curves for 6 compounds was completed.
2. Acceptable indices for the different stages of the transformation process were developed; i.e. Morphological indices, enzyme markers, growth in soft agar, production of tumor in the mice.

II. a) Work Scope: 1978

A. To examine the cytotoxic effects of the polynuclear hydrocarbons and hydrazines in jet fuel have on relative cloning efficiencies of human cells in vitro.
B. To score the cultures for morphologically abnormal clones, (morphological transformation).
C. To serially subpassage the abnormal colonies beyond their normal lifespan and then clone in soft - agar followed by inoculation of a bolus of cells into a xenogeneic immunologically suppressed host to test for the malignant potential.
D. To synchronize human cells in vitro and treat synchronized cells with suspected chemical carcinogens during different phases of cell cycle, (G0, G1, S, G2, M).
E. To investigate changes in DNA unscheduled repair kinetics after treatment of the cells in G0 and G1 cell cycling phases of the cell cycle.
F. To study changes in DNA directed DNA polymerase I and II activity after treatment of the cells with the carcinogens during the time when DNA repair is taking place in unsynchronized and synchronized cell populations.


It has been found that human cells can be transformed in vitro by a number of laboratories in the U.S. (Kakunaga, T., at N.C.I., Kakunaga, Cold Spring Harbor Symposium pg. 1537-1548, 1977; Kakunaga, T. Proc. Nat. Acad. of Sci., 1978; Milo, G., and D'ipalo, Nature 115:130-132, 1978). At present other laboratories have been successful also in learning how to transform human cells, (Vogelstein at John Hopkins; McClosky at Northrop laboratories in Research Triangle Park; Maher at M.S.U. at East Lansing Michigan;
Zimmerman at Harvard University). The procedure we have determined is effective in studying chemical carcinogen induced transformation was presented in the progress report for the periods covering July 1, 1977 to March 1, 1978. The cytotoxicity graphs reported on last year have been expanded upon. The data accumulated at that time was by trypan blue dye exclusion, (Annual progress report 1977-1978 Section IX page 83). We now have extended these observations to a more sensitive procedure. However, the second procedure, cloning studies, takes up to three weeks to complete the experiments. Cytotoxicity data accumulated by this means has permitted us to more rigidly define the "transformation dose" to be used in the transformation treatment regimen. (Table 1). We have expanded these results to include carcinogenicity data, (Table 1). The procedure used to determine carcinogenicity data will be presented in the text of this year's report, (1979).

III. b) Progress in 1979

Table 1

<table>
<thead>
<tr>
<th>Suspect Chemical Carcinogen</th>
<th>Concentration (ug/ml)</th>
<th>Carcinogenic*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzo (a) Pyrene</td>
<td>&gt;10</td>
<td>+</td>
</tr>
<tr>
<td>Benzo Pyrene-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8. diol 9,10 epoxide I (anti)</td>
<td>0.034</td>
<td>+</td>
</tr>
<tr>
<td>α-naphylamine</td>
<td>65</td>
<td>+</td>
</tr>
<tr>
<td>β-naphylamine</td>
<td>10</td>
<td>+</td>
</tr>
<tr>
<td>N=0-α-naphylamine</td>
<td>28</td>
<td>+</td>
</tr>
<tr>
<td>N=0-β-naphylamine</td>
<td>23</td>
<td>+</td>
</tr>
<tr>
<td>N=OH-α-naphylamine</td>
<td>2</td>
<td>+</td>
</tr>
<tr>
<td>N=OH-β-naphylamine</td>
<td>1.7</td>
<td>+</td>
</tr>
<tr>
<td>α-NO2-naphylamine</td>
<td>42</td>
<td>-</td>
</tr>
<tr>
<td>β-NO2-naphylamine</td>
<td>33</td>
<td>-</td>
</tr>
<tr>
<td>monomethylhydrazone</td>
<td>62.0</td>
<td>-</td>
</tr>
<tr>
<td>U.D.MH (1,1 DMH)</td>
<td>N.T.</td>
<td>+</td>
</tr>
<tr>
<td>1,2 DMH</td>
<td>N.T.</td>
<td>-</td>
</tr>
<tr>
<td>hydrazine</td>
<td>35</td>
<td>+</td>
</tr>
<tr>
<td>Methylazoxymethanol</td>
<td>0.005</td>
<td>-</td>
</tr>
<tr>
<td>bis UDMH (tetrazine)</td>
<td>50.0</td>
<td>+</td>
</tr>
<tr>
<td>phenylhydroaine</td>
<td>16.00</td>
<td>-</td>
</tr>
<tr>
<td>Methylazoxymethanol acetate</td>
<td>3.6</td>
<td>+</td>
</tr>
</tbody>
</table>

*Carcinogenicity is defined as the ability of the transformed cell populations to produce a tumor in a nude mouse 4-6 weeks after inoculation of the treated cells.

c) Other objectives and progress achieved in 1978-1979 are as follows:

I. We developed a protocol for evaluating suspect chemical or physical carcinogens on human cells, (Figure 1). Once Stage 1 and 2 are complete we then begin stage 3, TREATMENT PROTOCOL. Figure 2 illustrates how we treat human cells in vitro to induce a transformation event.
The induction stage is composed of substages, i.e. activation and/or direct action of the carcinogen. Carcinogens requiring activation such as Benzo(a)Pyrene are added at 24 hour and left on until hour 44. Carcinogens not requiring activation such as B[a]P-diol-epoxide I (anti) or N-OH-naphylamines were added to the cells in early S (33 hour-34 hour).

Once the induction stage is complete we have determine that a progression of events (time) must elapse before different stages of expression of the transformed phenotype will be expressed (Figure 3).

Under each stage of expression, (Figure 3) we have listed indices that have retained a high correlation of reliability for 23 different carcinogen treated populations.

Figure 3
Table 1

Indices used to determine the transformed state of carcinogen treated normal human cells in vitro

1. Cell growth from low cell densities after 1:10 splits. (Early)
2. Shortened population doubling times. (Early)
3. Indefinite extension of lifespan. (Late)
4. Loss of density dependent inhibition when high cell densities were reached. (Early)
5. Cells in colonies seeded at low cell density from criss-cross disoriented piled up non-contact inhibited colony morphology. (Early only)
6. Cells exhibit change from fibroblastic shape in morphology to short polygonal multinucleate architecture. (Early only)
7. Cell growth in air atmosphere without CO₂ enrichment at 37°C. (Early)
8. Cell growth in 1% fetal bovine supplemented 1x growth medium. (Early)
9. Cell growth at 41°C up to 144 hours while normal cells did not survive a 24 hour treatment. (Early)
10. Cell growth in 0.3% agar supplemented with 1x growth medium low in calcium. (Transitional).
11. Cell growth in 1% agarose supplemented with 1x growth medium. (Transitional).
12. Different agglutination pattern of the transformed cells compared to normal untreated cells (Wheat germ agglutination). (Early)
13. Differential tolerance to selective chemical compounds and complexes indicative of the transformed state; e.g., heparin tolerance, ouabain sensitivity, dextran sulfate tolerance. (Transitional)
14. Differential response of transformed cells to tumor cell lysates. (Transitional)
15. Growth in nu/nu mouse, 4–6 weeks. (Late)
16. Growth on a embryonic chick skin organ culture explant (3 days). (Late)

Other indices are listed in Table

1. Index number 16 is presently under evaluation. To date we have a 100% correlation with mouse tumor data.
2. Early events in the transformation process are sometimes difficult to detect and in vitro indices are used to differentiate transformed cell populations from normal cell populations. The agglutinin we chose in this case was wheat germ lectin. The following results were obtained for the following transformed cell populations, Table 2,3.
3. One of the procedures we use to select for transformed cell populations before injection into nude mice is to serial passage the cells through soft agar. If they passage through soft agar twice, they will, as a rule, grow in nude mice, Table 4.

We have defined an adequate number of indices that can be associated with changes associated with the transformation process at the cellular level.
**Table 2. - Resistance of Normal or Transformed Cells to Heparin or Dextran Sulfate.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Passage Level</th>
<th>Cloning Efficiency</th>
<th>Heparin (µg/ml)</th>
<th>Dextran Sulfate (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>400 100 25 6</td>
<td>80 20 5 1</td>
</tr>
<tr>
<td>None</td>
<td>32</td>
<td>3</td>
<td>7b 15 27 101</td>
<td>2 8 24 81</td>
</tr>
<tr>
<td>AF-81</td>
<td>23</td>
<td>7</td>
<td>3 24 73 95</td>
<td>4 16 73 94</td>
</tr>
<tr>
<td>N-Ac-AAF</td>
<td>23</td>
<td>6</td>
<td>4 47 86 83</td>
<td>3 19 68 104</td>
</tr>
<tr>
<td>PrS</td>
<td>25</td>
<td>6</td>
<td>4 55 95 110</td>
<td>4 28 93 112</td>
</tr>
<tr>
<td>4-NQO</td>
<td>36</td>
<td>5</td>
<td>7 57 58 68</td>
<td>5 5 8 70</td>
</tr>
<tr>
<td>s-PL</td>
<td>46</td>
<td>8</td>
<td>19 84 98 100</td>
<td>8 10 18 74</td>
</tr>
<tr>
<td>1-NA</td>
<td>53</td>
<td>8</td>
<td>40 89 93 84</td>
<td>5 9 28 97</td>
</tr>
<tr>
<td>2-NA</td>
<td>60</td>
<td>9</td>
<td>24 108 127 130</td>
<td>1 2 7 122</td>
</tr>
</tbody>
</table>

*Human cell populations transformed by N-Ac-AAF, PrS, 4NQO, s-PL, 1-NA, 1-NA or Af-81 serially passaged to PDL 20. The transformed cells were passaged through soft agar (0.33%), repopulated and used during the transitional stage. The control cells (noncarcinogen treated) were serially passaged to comparable PDL.*

*Percent survivors, based upon the cloning of untreated cells. N.D.=not done.*
Table 3. - Resistance of Normal of Transformed\textsuperscript{a} Cells to KB Cell Lysate.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Passage Level</th>
<th>Cloning Efficiency</th>
<th>KB Lysate (Cell Equivalent X 10^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>5</td>
<td>6</td>
<td>10^6 9 103 94 100 98</td>
</tr>
<tr>
<td>None</td>
<td>32</td>
<td>3</td>
<td>N.D. N.D. 32 68 85 96</td>
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<tr>
<td>AF-B\textsubscript{1}</td>
<td>23</td>
<td>/</td>
<td>37 78 51 91 85 81</td>
</tr>
<tr>
<td>N-Ac-AAF</td>
<td>23</td>
<td>6</td>
<td>8 64 83 102 101 99</td>
</tr>
<tr>
<td>PrS</td>
<td>25</td>
<td>6</td>
<td>33 67 71 95 106 102</td>
</tr>
<tr>
<td>4-NQO</td>
<td>36</td>
<td>5</td>
<td>N.D. N.D. 60 76 81 97</td>
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<tr>
<td>(\beta)-PL</td>
<td>46</td>
<td>8</td>
<td>N.D. N.D. 85 102 105 95</td>
</tr>
<tr>
<td>1-NA</td>
<td>53</td>
<td>8</td>
<td>N.D. N.D. 98 97 97 89</td>
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<tr>
<td>2-NA</td>
<td>60</td>
<td>9</td>
<td>N.D. N.D. 112 121 93 115</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Human cell populations transformed by N-Ac-AAF, PrS, 4NQO, \(\beta\)-PL, 1-NA, 1-NA or AF-B\textsubscript{1} serially passaged to PDL 20. The transformed cells were passaged through soft agar (0.33%), repopulated and used during the transitional stage. The control cells (noncarcinogen treated) were serially passaged to comparable PDL.

\textsuperscript{b} Percent survivors, based upon the cloning of untreated cells. N.D. = not done.
Table 4. - Characteristics of Transformed Human Skin Fibroblasts During Transition from the Early Periods of the Transformation Process Through to Neoplasia.

<table>
<thead>
<tr>
<th>Carcinogen</th>
<th>E.D. 50&lt;sup&gt;a&lt;/sup&gt;</th>
<th>L.A.&lt;sup&gt;b&lt;/sup&gt;</th>
<th>S.A.&lt;sup&gt;c&lt;/sup&gt;</th>
<th>T.I.&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-PL</td>
<td>13.0</td>
<td>N.D.&lt;sup&gt;e&lt;/sup&gt;</td>
<td>14.0</td>
<td>3/4</td>
</tr>
<tr>
<td>AF-B&lt;sub&gt;1&lt;/sub&gt;</td>
<td>10.0</td>
<td>78</td>
<td>10.0</td>
<td>8/14</td>
</tr>
<tr>
<td>PrS</td>
<td>5.0</td>
<td>125</td>
<td>20.0</td>
<td>7/11</td>
</tr>
<tr>
<td>4-NQO</td>
<td>0.002</td>
<td>39</td>
<td>0.1</td>
<td>2/4</td>
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<tr>
<td>MNNG</td>
<td>0.5</td>
<td>39</td>
<td>1.0</td>
<td>3/5</td>
</tr>
<tr>
<td>EMS</td>
<td>10.0</td>
<td>N.D.</td>
<td>20.0</td>
<td>2/4</td>
</tr>
<tr>
<td>1-NA</td>
<td>65.0</td>
<td>19</td>
<td>1.5</td>
<td>2/9</td>
</tr>
<tr>
<td>2-NA</td>
<td>68.0</td>
<td>250</td>
<td>0.1</td>
<td>1/6</td>
</tr>
<tr>
<td>N-OH-1-NA</td>
<td>2.0</td>
<td>N.D.</td>
<td>30.0</td>
<td>1/8</td>
</tr>
<tr>
<td>N-OH-2-NA</td>
<td>1.7</td>
<td>N.D.</td>
<td>29.0</td>
<td>2/7</td>
</tr>
<tr>
<td>N-O-1-NA</td>
<td>27.5</td>
<td>19</td>
<td>5.1</td>
<td>3/16</td>
</tr>
<tr>
<td>N-O-2-NA</td>
<td>23.1</td>
<td>250</td>
<td>1.0</td>
<td>3/16</td>
</tr>
<tr>
<td>N-Ac-AAF</td>
<td>0.5</td>
<td>39</td>
<td>0</td>
<td>0/8</td>
</tr>
<tr>
<td>Hydrazine</td>
<td>35.0</td>
<td>19</td>
<td>9.2</td>
<td>4/8</td>
</tr>
<tr>
<td>UDMH</td>
<td>50.0</td>
<td>N.D.</td>
<td>5.1</td>
<td>4/8</td>
</tr>
<tr>
<td>MAMA</td>
<td>3.6</td>
<td>N.D.</td>
<td>900.0</td>
<td>2/16</td>
</tr>
<tr>
<td>B(a)P</td>
<td>10.0</td>
<td>39</td>
<td>1.0</td>
<td>6/10</td>
</tr>
<tr>
<td>MMS</td>
<td>0.1</td>
<td>2500</td>
<td>0</td>
<td>0/6</td>
</tr>
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<td>U.V.</td>
<td>40 J.m&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>78</td>
<td>20.0</td>
<td>4/6</td>
</tr>
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<td>137Cs</td>
<td>100 r</td>
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<td>13.1</td>
<td>3/7</td>
</tr>
<tr>
<td>Control</td>
<td>---</td>
<td>2500</td>
<td>0</td>
<td>0/10</td>
</tr>
</tbody>
</table>
Legend - Table 4.

The ED 50 of these carcinogens was determined prior to and following the induction phase of the transformation protocol. The Toxicity values presented here were obtained in the pretreatment phase.

These values were obtained using wheat germ agglutinin (L.A. = lectin agglutination).

Fifty thousand cells at PDL 20 were seeded into 0.33% agar (S.A.) supplemented with LoCal + 20% FBS, overlaid on a 2.0% agar base supplemented with RPMI-1629 + 20% FBS. The colonies were counted after 21 days. Frequency as expressed in column c (S.A.) is the number of colonies formed 28 days after seeding per number of cells seeded. The values were normalized to one hundred thousand cells.

The tumor incidence (T.I.) is expressed as a fraction: the numerator is the number of mice giving rise to tumors 0.8 - 1.2 cm in size, 4-6 weeks after the injection of 5 × 10^6 cells and the denominator is the total number of preirradiated (450 rats, whole body) mice injected with a given cell population.

N.D. = Not determined.

Text Continued

The indices we have selected as of primary importance to use are presented in Table 4. Our reasons for deciding on these are: 1) The early stage in the process can be readily identified by altered lectin agglutination profiles. Moreover, recently these lectins are available with an F.I.T.C. label attached and the amount of binding can be quantified.

Moreover the growth of transformed cells in soft agar a measure of the state of anchorage independence can be quantified. Lastly, tumor production in the mouse can be quantified. The main drawbacks to the nude mouse is 1) positive tumor formation occurs in 4-6 weeks, however negative results are not scoreable within 1 year, 2) variability in resistance to susceptibility in nu/nu mice from different backgrounds, 3) interference in tumor takes by the presence of indigenous murine viruses, 4) cost of housing.

Included under this cover is a photograph of colonies (UDMH-transformed cells) growing in soft agar.
Colony(s) of UDMH transformed human skin fibroblasts 3 weeks after seeding growing in 0.33% agar. These colonies are removed and then placed in nude mice (Figure 5). After 6 weeks the tumors are excised (Figure 6B) and submitted for histopathology.

III. Presentations, Abstracts - FY-1979


IV. Papers Published FY-1979 Sponsored by A.F.S.O.R.


VI. Papers Submitted 1980 Sponsored by A.F.S.O.R.


Principal Experiments in Progress with Other Laboratories

We are presently concluding work with Dr. Fred Kadlubar at the National Center for Toxicological research on the activity of the amines and their derivatives on the interaction of the amines and DNA. Another collaboration that will bear fruition presently will be our work with Dr. Allen Jefferies at the College of Physicians and Surgens Comprehensive Cancer Center at Columbia University. We are examining the relationship between chemical carcinogen induction and adduct formation. Our work with Dr. Donald Witiak is beginning to bear results. We are assisting his laboratory in two ways. First, we are studying the relationship between the hydrazine compounds and derivatives with the induction of carcinogenesis and adduct formation. Secondly, we are using the radiolabeled hydrazine carcinogens in our system to study the events that occur during the expression phase of carcinogenesis. This is being accomplished by studying the interaction of the hydrazines with the acid soluble histone and non-histone nuclear proteins. We have concluded the radiolabeling studies and are finishing up the studies designed to quantitate the changes in histones during the early and transitional stages of the carcinogenesis process.

Our work on the translocation of benzo(a)pyrene into the human cells is continuing. We have determined that the cytoplasmic protein complex is required to transport the BP into the nucleus. We have identified the protein complex to be a lipoprotein of a molecular weight value of 12,500. We are presently characterizing the complex.
Principal Experiments and Time Schedule for the Main Objectives for FY-80

The objectives mentioned on page 12 in the 1979 annual report have been accomplished with the following exception(s). Due to the deletion from last year's budget for D. L. Allred salary and supplies we deleted the E.M. work. Another change we have made in the interest of time for evaluating the neoplastic stage of the transformation process is to use a chick embryonic skin organ culture. The methodology for the organ culture was worked out in this laboratory. The chief advantage to using this system is a reduction in time to evaluate neoplastic transformation. We need only 3 days to evaluate neoplasia with the chick skin system whereas we need 4-6 weeks to evaluate tumor formation in the nude mouse. (Renewal Proposal 1980). In one of the manuscripts accepted in the International Journal of Cancer we have examined specific compounds for their modulating effects for presensitizing human cells to the subsequent induction of carcinogenesis in the presence of the chemical carcinogens. We have just completed carcinogen induction experiments on the human skin epithelial system. We will be publishing on this in the fall. Our work is going along well in our collaborative effort with Dr. Witjak.

We are continuing our experimentation on the studies examining changes in histone labeling patterns during the different stages of the carcinogenesis process.

IX. Support Data 16 Copies of each document.
Figure 2

TREATMENT PROTOCOL

% Labeled Interphases

CELL CYCLE

COMPLETE MEDIUM
INSULIN
(NON-PROLIFERATING MEDIUM)
(-ARG, -GLUT)
Carcinogen Added
Carcinogen Removed

TIME (hr)

0 8 16 24 32 40 48
Figure 3

SELECTION OF TRANSFORMED PHENOTYPE

<table>
<thead>
<tr>
<th>EARLY</th>
<th>TRANSITIONAL</th>
<th>LATE</th>
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<td>5 wks</td>
<td>0.33% Soft Agar</td>
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Figure 4
Characteristics of Cytoplasmic Polynuclear Hydrocarbon Binding Protein

R. Tejwani and G. E. Milo

When proliferating human diploid foreskin cells were treated with \(^3\)H-B(a)P \textit{in vitro}, the radiolabel was found associated with a cytoplasmic protein. Optimum binding of the \(^3\)H-B(a)P occurred after twelve hours. Twelve hours later the radiolabel was localized in the nucleus. The \(^3\)H-B(a)P was bound to a protein of molecular weight 12,500, as determined by Sephadex G-200 chromatography. Organic extraction and separation of the radiolabelled polynuclear hydrocarbon from the complex followed by high pressure liquid chromatographic analysis of the B(a)P fraction indicated that the parent B(a)P molecule, and not the polynuclear hydrocarbon metabolites, was bound to the protein. Non-proliferating cells, which contained inducible functional AHH activity, did not bind or transport the \(^3\)H-B(a)P into the cytoplasm. The events associated with binding and movement of \(^3\)H-B(a)P into the nucleus subsequently led to the induction of a carcinogenic response by the treated human diploid cell population.

Supported in part by the National Cancer Institute NO 1-CP-43276 and Air Force Office of Scientific Research F 49620-77-C-0110.

Altering incubation conditions from a neutral to a slightly acidic pH causes an 18-fold increase in the in vitro binding of N-hydroxy-1-naphthylamine (N-OH-1-NA) to calf thymus DNA (Kadlubar et al., Cancer Res. 38: 3628, 1978). Therefore, we studied the effect of pH on the in vitro transformation of normal human cells induced by N-OH-1-NA or N-OH-2-NA. Low passage human fibroblasts were blocked prior to the G1/S interphase by amino acid deprivation, released and then treated during the early S phase of the cell cycle with 2.0 μg/ml N-OH-1-NA or N-OH-2-NA for 15 minutes (under argon) in serum-free medium at either pH 5.0 or pH 7.0. Affected cells were then selectively propagated in growth medium containing 8X nonessential amino acid and 2X vitamins. A comparison of the frequency of growth in soft agar (0.3%) demonstrated a 7-fold increase in N-OH-1-NA-induced bolus formation with cells treated at pH 5.0 (1:102.88) over those treated at pH 7.0 (1:103.72). Similarly, bolus formation from cells treated with N-OH-2-NA at pH 5.0 (1:103.02) was 4-fold higher than those treated at pH 7.0 (1:103.63). The conversion of the N-OH arylamines to an arylnitrenium ion or carbocation is favored at pH 5.0 and the acidic conditions normally found in the bladder lumen would also favor the formation of these electrophilic ions. Therefore, these results further implicate the N-OH arylamines as having a significant role in arylamine-induced urinary bladder carcinogenesis. (This work was supported in part by Air Force Office of Sci. Research F49620-77-C-0110)
BENZO(a)PYRENE METABOLISM BY TRANSFORMABLE HUMAN SKIN FIBRO-BLASTS. Raman Tejwani*, Ronald N. Trewyn*, and George E. Milo. The Ohio State University, Columbus, Ohio 43210.

The metabolism of benzo(a)pyrene (BP) to reactive intermediates by microsomal mixed-function oxidases (MFO's) is considered to be important in the induction of carcinogenic events in mammalian cells. Human skin fibroblasts can be transformed in vitro by BP (Cancer Res. 38: 3026, 1978), so these cells are being used to study the role of MFO's in the transformation of human cells. Profiles of intracellular and extracellular metabolites of BP have been examined using high performance liquid chromatography to quantitate oxygenated products of the MFO's. After treatment, the primary intracellular (nuclear) polycyclic aromatic hydrocarbon (PAH) is the parent compound BP. Extracellular metabolites account for less than 10% of the added (0.1 μM) PAH after 24 hours, with phenolic derivatives representing the major (2%) oxygenated form. BP tetrols (diol epoxides), diols, and quinones account for less than 1% of the remaining metabolites. The limited generation of oxygenated intermediates by microsomal MFO's suggests that these enzymes play an alternate or indirect role in the transformation of human skin fibroblasts by BP. Other enzyme-derived, organic-extractable metabolites of BP, possibly methylated derivatives, are being evaluated for their role in the transformation process. (Supported in part by NIH R-01-CA-25901-01; Air Force F-49620-77-C-0110).

All compounds that are designated by code or initial letters must be identified adequately in the abstract, e.g., MJ-1999: 4-(2-isopropylamino-1-hydroxyethyl) methanesulfonanilide hydrochloride.

Society Affiliation
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Each Abstract Form submitted MUST BE SIGNED by a member of the American Society of Biological Chemists or Biophysical Society (Indicate Affiliation in box at left)

George E. Milo
(Member's Name: Please Print or Type Full Name)

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Telephone No.: Area Code. 614..#.422-1478
Requirements for the induction of carcinogenesis in human cells for different classes of Jet Fuel Components. George E. Milo, James W. Oldham and Raman Tejwani. Department of Physiological Chemistry and Veterinary Pathobiology, The Ohio State University, Columbus, Ohio 43210

Recent data reported at the Ninth Annual Environmental Toxicology Meeting held in Dayton indicated that hydrazine and UDMH could initiate early events in the carcinogenesis process in human cells leading to tumor growth of these cells in a suitable assay system. The tumors, when removed from the mice and analyzed in vitro by G-banding, carried karyotypic markers suggesting a change in the heterochromatin. Recent evidence indicates that compound requiring enzymatic activation such as benzo (a) pyrene B(a)P are specifically transported to the nucleus as parent B(a)P by a carrier protein complex. The B(a)P is then activated in the nucleus and the metabolites interact with the DNA. Results from adduct studies indicate that the 7,8-9, 10 endiol epoxide I metabolite interacts with the guanosine residue in DNA. Other compounds such as N-OH arylamines that do not require enzymatic activation or specific transport into the nucleus need only a 15 minute treatment period when the cells are in S phase of the cell cycle to induce a carcinogenic response. This response is enhanced by treating at an acid pH where the formation of an electrophilic arylnitrenium is favored. Hydrazine and UDMH do not require activation and have a very short half life in the growth medium, approximately 2-5 minutes. Effective carcinogenic responses occur when the cells are treated during early S. Therefore we have identified specific requirements for activation, transport and molecular interaction that are different for each class of carcinogen.

Our immediate plans are to:

1) use chemical analogues of hydrazine or UDMH to study the mechanism of the carcinogenesis process

2) investigate the roles plasma membrane associated aryl-hydrocarbon hydroxylase (AHH) activation and nuclear associated AHH have-in-the detoxification and/or carcinogenesis process

3) continue characterizing the metabolite profiles and movement of BP into the cell

4) study events immediately following carcinogen localization in the nucleus, i.e. post-translational modification of histones and alterations in DNA polymerase activities.
Kinetics of Movement of Benzo(a)pyrene into Transformable and Non-Transformable Human Diploid Cells

R. Tejwani, R. W. Trewyn and G. E. Milo

Benzo(a)pyrene (B(a)P), an environmental pollutant, can transform low passage (<PDL 6) human skin fibroblasts in vitro (Cancer Res. 38: 3026, 1978). High passage cells (>PDL 20) are resistant to transformation by this carcinogen. B(a)P binds differentially to cytoplasmic protein complexes in the transformable and refractory cells (Cancer Letters, in press). In the transformable cells, the movement of the B(a)P to the nucleus is coincident with the optimum time for induction of carcinogenesis; i.e., 12-24 hours post-treatment. Therefore, low and high passage cells were treated with [G-^3H]B(a)P for 12 hours followed by exposure to unlabeled B(a)P for up to 96 hours. This pulse-chase experiment was designed to follow the distribution of the B(a)P and/or B(a)P metabolites in the cytoplasm, nucleus, and extracellular growth medium over the course of 96 hours. High performance liquid chromatograpy profiles of the intracellular and extracellular, ethyl acetate extractable fractions demonstrated that >75% of the B(a)P remained unmethylated in sensitive and refractory cells. The uptake of B(a)P into the nucleus of the non-transformable cells was approximately 50% less than the transformable cells at most time points. Also, the non-transformable cells removed the B(a)P from the nucleus to the extracellular environment more rapidly over the 96 hr time period. The refractory nature of the high passage cells may be explained by the differential binding of B(a)P to cytoplasmic proteins, the diminished transport of B(a)P to the nucleus, and/or the more rapid removal of B(a)P localized in the nucleus.

Supported in part by RO1-CA-25907 and A.F.S.O.R. F49620-77-C-0110.
ANALYSIS OF INTRACELLULAR DISTRIBUTION AND BINDING OF BENZO[a]PYRENE IN HUMAN DIPLOID FIBROBLASTS

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SUMMARY

Previous work with low passage synchronized human foreskin fibroblast cell populations has indicated that benzo[a]pyrene (BP) can induce a carcinogenic event [3]. BP additionally has shown to damage DNA in logarhythmically growing low passage cultures [9]. High passage cells, on the other hand, seem to be refractory to transformation by BP, even though this agent can induce DNA damage, similar to that seen in low passage cells. When low passage cells were treated with BP, the initial binding of the hydrocarbon was primarily to a cytoplasmic protein complex of molecular weight 12,500, while in high passage cells, a major portion of BP was bound to a protein complex of molecular weight 200,000. High-pressure liquid chromatography (HPLC) profiles of ethyl acetate extractable fractions from the BP-cytoplasmic protein complexes of low and high passage cells demonstrated that the majority of the BP remained unmetabolized. When nuclei were isolated from low and high passage cells prior to the HPLC analysis, the major component (90%) was again unmetabolized BP. The results suggest selective attachment of BP to different cytoplasmic protein...

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*To whom request for reprints should be addressed.

Abbreviations: PNH, polynuclear hydrocarbon; BP, benzo[a]pyrene; HNF, human neonatal foreskin; HPLC, high pressure liquid chromatography; Buffer A, 0.03 M Na phosphate — 0.03 M Tris—HCl — 2.5 mM Na, EDTA — 0.5 mM DTT (dithiothreitol), pH 7.5; Buffer B, 0.01 M Tris maleate — 1 mM DTT — 3 mM Ca (Ac), — 2 mM Mg (Ac), pH 7.5; BHT, butylated hydroxytoluene; BP-9, 10-diol, 9,10-dihydro-9,10-dihydroxy BP; BP-4, 5-diol, 4,5-dihydro-4,5-dihydroxy BP; BP-7, 8-diol, 7,8-dihydro-7,8-dihydroxy BP; BP-11, 12-diol, 11,12-dihydro-11,12-dihydroxy BP.
complexes of logarithmically growing human diploid fibroblast cells dependent on the passage level of the cells.

INTRODUCTION

Polynuclear hydrocarbons (PNH) are a class of molecules which must be activated to reactive metabolites in order to function as mutagens or carcinogens. This activation involves the conversion of polynuclear hydrocarbons (PNH)\(^*\), such as BP, to dihydrodiols, oxides, phenols, quinones and water-soluble conjugates [4-6,12,13]. The 7,8-dihydrodiol-9,10-oxide of BP has been shown to be the major reactive metabolite bound to DNA [14]; BP 7,8-dihydrodiol-9,10 oxide (anti) deoxyguanosine is the major DNA adduct formed in human and bovine bronchial explant tissue [7]. The formation and cellular processing of this covalent DNA adduct is suspected to be a crucial event in BP-induced carcinogenesis.

Earlier reports from our laboratory have shown that BP absorbed into human neonatal foreskin (HNF) cells in culture and first accumulates in the cytoplasm [3]. Labelling of the cells with L-[4,5\(^{3}H\)]leucine before treatment with [7,10-\(^{14}C\)]BP indicated that the PNH is initially bound to a cytoplasmic protein complex (unpublished data). Distribution of the BP into the nucleus of treated cells occurs 12 h later [3]. Moreover, BP treatment of HNF cells neoplastically transforms them at passage level 5 (low passage cells), but does not transform cells above passage level 10 (high passage cells) [8]. Optimum transformation is observed when low passage cells are treated with BP 12-24 h prior to entering the S phase; this treatment causes 3.0 breaks/10\(^{11}\) daltons of DNA [9]. The time required for optimum BP-induced DNA damage coincides with the specific time period in which optimum BP enhancement of focus formation occurs in SV-40 infected transformable cells, i.e., treatment of the cells with BP 12-24 h prior to infection has been shown to enhance focus formation 2-fold [11]. Since SV-40 (viral) DNA does not need to replicate in order to be integrated into the host cell DNA, these observations support the concept that parent (unmetabolized) BP is directly involved in the transformation process within the nucleus. This report presents studies on the nature of association of BP with the cytoplasmic protein complex and nuclei from low and high passage cells, 12-24 h following the initiation of treatment.

MATERIALS AND METHODS

Preparation and isolation of BP-cytoplasmic protein complex

All extraction procedures were carried out under argon and red light to reduce photoxidation and autoxidation.

Passage 5-25 HNF cells were grown, serially subpassaged, and treated with BP as described earlier [3]. After seeding 48-72 h (40-60% cell confluency), the growth medium was replaced with a carcinogen-supple-
mented medium containing 0.125–26 μM [G-3H]BP (16–40 Ci/mmol). After 12 h of treatment, the cells were harvested and washed. The 650 × g cell pellet [3] was homogenized in 2 ml of Buffer A and centrifuged at 100,000 × g for 1 h. The cytoplasmic fraction was partitioned with dextran-coated charcoal in Buffer A, and 2 ml (0.2–0.3 mg/ml protein) of the [G-3H]BP protein complex was applied to a Sephadex G-200 column (58 × 0.9 cm). Elution of 0.5 ml fractions was carried out with 50 ml of Buffer A at a flow rate of 15 ml/h. An aliquot of each fraction was removed and the radioactivity assayed in a Packard Tri-Carb liquid scintillation counter at a tritium counting efficiency of 38%.

**HPLC of [G-3H]BP metabolites**

Preconfluent cells were exposed to 0.072 μM [G-3H]BP (1 mCi/ml, 27 Ci/mmol) for 12 h and a 100,000 × g fraction was prepared as described above. For the nuclear metabolite profiles, cells were treated with 26 μM [G-3H]BP (1 mCi/ml, 27 Ci/mmol) for 24 h, and nuclei were prepared by a modification of the procedure of Chaveau et al. [2]. The nuclear pellet was suspended in Buffer B and contrast-interference Nomarski microscopic examination of the nuclear suspension indicated a 35% recovery of nuclei.

Extraction of either the BP-cytoplasmic protein complex or the nuclear fraction was completed with 3 vols. of ethyl acetate in the presence of 0.8 mg/ml BHT; the organic phase was passed over anhydrous sodium sulfate, filtered, dried under argon and dissolved in 0.5 ml of acetonitrile/methanol (2:1 v/v). Aliquots were removed for counting and the remaining sample was dried under argon and stored at −90°C. The sample was reconstituted with methanol/aacetone/DMSO (2:1:1 by vol.), non-radioactive BP metabolite standards added, and the extract chromatographed on a DuPont Instruments Model 848 High Pressure Liquid Chromatography with 4 mm × 30 cm μ-Bondapak C18 column (Waters Associates) using an isocratic elution solvent of methanol/water/ethyl ether (66.3:30.4:3.3, by vol.) at a flow rate of 1.4 ml/min. The effluent was monitored by UV spectrometry to identify metabolites, which were quantitated by collecting appropriate fractions of the effluent for liquid scintillation analysis. Six second fractions were collected for 11–12 min, then 12-s fractions were collected for 8–9 min and lastly, 60-s fractions were collected until the completion of the chromatographic run. Typical retention times in minutes for each metabolite were: BP-9,10-diol, 3.6; BP-4,5-diol, 5.5; BP-11,12-diol, 5.8; BP-7,8-diol, 6.5; BP-1,6-quinone, 9.1; BP-3,6-quinone, 9.6; BP-6,12-quinone, 11.0; BP-9-phenol, 15.5; BP-3-phenol, 19.1; BP, 37.5. The overall recovery of radioactivity from the column was greater than 90%.

**RESULTS**

[G-3H]BP-protein complexes isolated from the cytoplasm of low and high passage cells were chromatographed on a Sephadex G-200 column.
Fig. 1. Sephadex G-200 chromatography of a [G-3H]BP-cytoplasmic protein complex from low passage cells. The cytoplasmic fraction was prepared from passage 5 HNF cells treated with [G-3H]BP for 12 h. 50,000 dpm were applied to a Sephadex G-200 column (58 x 0.9 cm) and eluted with 0.01 M Na phosphate — 0.2 M Tris-HCl — 2.5 mM Na,EDTA — 5 mM DTT (pH 7.5). 0.5-ml fractions were collected and the radioactivity was assayed. Blue Dextran — 200,000; Aldolase — 158,000; Bovine Serum Albumin — 67,000; Hen Egg Albumin — 45,000; Chymotrypsinogen — 25,000; and Cytochrome c — 12,500 served as molecular weight standards.

Fig. 2. Sephadex G-200 chromatography of a [G-3H]BP-cytoplasmic protein complex from high passage cells. The cytoplasmic fraction was prepared from passage 25 HNF cells treated with [G-3H]BP for 12 h. 100,000 dpm were applied to a Sephadex G-200 column (58 x 0.9 cm) and 0.5 ml fractions were collected as described under Fig. 1.
In low passage cells, a major portion of BP was associated with a protein complex of molecular weight 12,500. The ratio of area under the low molecular weight peak to the high molecular weight peak was 6.5 (Fig. 1). In high passage cells, a major portion of BP was associated with a protein complex of molecular weight 200,000 and the ratio of the area under the

![HPLC profiles](image)

Fig. 3. HPLC profiles of the ethyl acetate extractable radioactivity from the total cytoplasmic protein-hydrocarbon complexes of low and high passage cells. Non-radioactive BP metabolite standards were cochromatographed with the radioactive extract for metabolite identification. An isocratic elution solvent of methanol/water/ethyl ether (56:3:30.4:3.3, by vol.) was employed at a flow rate of 1.4 ml/min and fractions collected for liquid scintillation spectrometry. (See Material and Methods for details.) Upper panel: Profile of the organic extract of [G-3H]BP-total cytoplasmic protein complex from high passage human diploid fibroblasts. A total of 6300 dpm were applied to the column. Lower panel: profile of the organic extract of [G-3H]BP-cytoplasmic protein complex isolated from low passage human diploid fibroblasts. A total of 4000 dpm were applied to the column. Note the discontinuity in the retention time scale.
high molecular weight peak to the low molecular weight peak was 1.8 (Fig. 2).

The total cytoplasmic protein complex isolated from low passage human fibroblast cells exposed to [G-H]BP was extracted with ethyl acetate, and the non-covalently bound hydrocarbon and its metabolites were co-chromatographed on a reverse phase column by high-pressure liquid chromatography with authentic reference standards. In the metabolite profiles of

![HPLC profile](image)

Fig. 4. HPLC profiles of the ethyl acetate extractable radioactivity from nuclei of low and high passage human diploid fibroblasts exposed to [G-H]BP. Non-radioactive BP metabolite standards were cochromatographed with the radioactive extract for metabolite identification. (See Fig. 3, Materials and Methods for details). Upper panel: profile of the organic extract from nuclei of high passage cells. A total of 1.263 × 10^6 dpm were applied to the column. Lower panel: Profile of the organic extract from nuclei of low passage cells. A total of 580,850 dpm were applied to the column. BP-11,12-diol and BP-11,12-quinone cochromatographed with BP-4,5-diol and BP-3,6-quinone, respectively.
BP-treated low passage cells (Fig. 3), unmetabolized BP was the only radio-labeled fraction. Similar results were obtained with BP radioactivity isolated from the total cytoplasmic protein complex of high passage cells, except that a small peak of radioactivity eluted prior to the BP-9, 10-diol (Fig. 3).

The radioactivity associated with isolated nuclei of low and high passage cells treated with [G-3H]BP was chromatographed as described above (Fig. 4). The nuclei from high passage cells had radioactivity which co-chromatographed with BP-1, 6:3,5 and 6,12-quinones and BP-9-phenol; 99% of the counts were associated with BP; however, the radioactivity isolated from [G-3H]BP-treated low passage cells co-chromatographed with BP-9-phenol, with 96% of the counts eluting with BP. The unknown peaks at 2.4 and 4.5 min represent void volume radioactivity (pre-BP-9,10-diol) and an unidentified metabolite, respectively. Co-chromatography with BP-11,12-diol and BP-11,12-quinone indicated that the 4.5 min peak represented neither of these potential metabolites.

DISCUSSION

When low passage HNF cells are treated with BP, the PNH accumulates in the cytoplasm, before localizing in the nucleus 24 h after exposure. Sephadex G-200 gel chromatographic separation of the cytoplasmic protein complexes from low passage and high passage cells indicated the distribution of BP between protein complexes of molecular weights, 12,500 and 200,000. In low passage cells, the amount of BP associated with the low molecular weight protein complex was 4–7 times that associated with the high molecular weight protein complex. However, in high passage cells, the amount of BP associated with the high molecular weight protein complex was 0.8–1.8 times that associated with the low molecular weight protein complex. HPLC analysis of the BP radioactivity separated from the BP-total cytoplasmic protein complex of low passage and high passage cells indicated that the major fraction was the parent BP. This is in contrast to a previous study [10], in which active metabolites of 3'-methyl-4-dimethyl amino azobenzene were shown to bind to a cytosol protein complex from rat liver. It was also interesting to observe that unmetabolised [G-3H]BP made up the major fraction of the PNH associated with the nuclei from low passage and high passage cells. The minor peaks observed in the HPLC metabolite profiles may be a result of autooxidation of the sample, although all procedures were performed under red light, argon, and in the presence of an antioxidant.

We have observed minor differences in the metabolites non-covalently bound to DNA, in low and high passage cells. Also, as described above, we have observed binding of BP to different cytoplasmic protein complexes in low and high passage cells. The transport of BP into the nucleus of these cells may involve an activation of the BP-cytoplasmic protein complex, similar to that observed with the steroids. Only binding of BP
to the lower molecular weight protein complex (the predominant complex in low passage cells) may result in an activation of the complex. Therefore, the accessibility of BP metabolites to specific nuclear binding sites in low and high passage cells may be different and may account for the susceptibility or refractoriness to BP-induced carcinogenesis of human fibroblast cells in vitro.

ACKNOWLEDGEMENT

The authors acknowledge the expert technical assistance of Betty Hyatt and Linda Montgomery. The authors thank Dr. Guido Daub for the generous sample of BP-11,12-diol and BP-11,12-quinone and the National Cancer Institute for the BP standards.

REFERENCES

GROWTH AND ULTRASTRUCTURAL CHARACTERIZATION OF PROLIFERATING HUMAN KERATINOCYTES IN VITRO WITHOUT ADDED EXTRINSIC FACTORS

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SUMMARY

Routine in vitro cultivation of human epithelial cells derived from foreskin and free of contaminating fibroblasts has been achieved without the addition of conditioned medium or extrinsic factors. Epithelial cell populations could be serially subpassaged and exhibited modulating responses at PDLs to culture conditions as the cells passed from phase 1 through phase 2 of their life span. The cell population in early phase 2 gave rise to tissue sheets that exhibited characteristics typical of human foreskin epidermis including the formation of distinct cellular layers, viz. strata basalis, spinocorn, granulosum and corneum. Typical keratohyaline granules were not observed in the epithelial cells although a distinct cornified layer was evident. Ultrastructurally, desmosomes and tonofilaments were readily apparent. Thus, the procedure detailed in this study will produce highly differentiated fibroblast-free epidermal sheets reaching several centimeters in size and which can be removed from the substratum as a single sheet of organized epidermis. The epithelial cells could be cultured through 20 ± 3 PDL, whereas fibroblast cultures derived from foreskin cultures exhibited 40 ± 5 PDL and mixed cell cultures of foreskin were carried through 43 ± 5 PDL.

Key words: epithelial cells; human skin cultures; skin epithelial cells; epidermis culture; ultrastructure of cultured epithelial cells.

INTRODUCTION

Reproducible in vitro cultivation of normal proliferating human epithelial cells has been difficult to achieve with present methodologies and the procedures developed to establish primary epithelial cell populations have resulted in a low rate of success (1–4). Enhanced establishment of epithelial cell populations from explants (5,6) occurs with the addition of fibroblasts or extrinsic growth factors, i.e. products released from cultured fibroblasts (conditioned growth media) (7), epidermal growth factor (8) or deoxyribonucleic acid (9), to the cultures or culture media. Recently, Freeman et al. reported that by the use of a dermal collagen bed derived from sterile pig skin, human epidermal cell cultures were established in 129 of 140 attempts; the cultured epithelial cells grew in the absence of fibroblasts or their products (except collagen). Earlier, we described a method for the enzymatic dispersion, growth and serial subpassage of primary cultures of human fibroblasts derived from foreskin (10); epithelial colonies were occasionally noted in these cultures after subpassage. We have now modified the culture technique in a manner that permits not only the establishment of pure populations of human fibroblasts but, more importantly, allows for the successful establishment of pure human epidermal epithelial cell cultures. The human epithelial cells were grown successfully without the addition of extrinsic growth factors or collagen substrata and have...
been serially subpassed, fibroblast-free, on a routine basis. Epithelial cells could be grown to form large multilayered epithelial-like sheets in which the cells showed differentiated characteristics consistent with those noted in normal intact epidermis for a limited number of PDLs (PDL 5).

It is the purpose of this report to detail the methodology used for the routine culture of human foreskin epithelial cells (keratinocytes), which grow to form large multilayer cell sheets. The growth characteristics and morphology of the cultured cells also will be described.

**Materials and Methods**

**Preparation of cell suspensions.** Human foreskin was obtained from infants at the time of circumcision. The epidermis was dissected carefully from the underlying connective tissue dermis and cut into 2-mm pieces in MEM-Hanks' balanced salt medium containing 25 mM HEPES buffer at pH 7.2 ([CM] culture medium). The tissue was rinsed three times in this medium and the tissue fragments transferred to 20 ml of CM medium supplemented with 20% fetal bovine serum (FBS) containing 0.25% collagenase (115 U per mg, 4197 CLS, Worthington Biochemical Corp., Freehold, New Jersey). Enzymatic tissue dispersion was done at 37°C in a 4% CO2-enriched air atmosphere for 5 hr or overnight. Cells were recovered from suspension by centrifugation at 150 × g for 7 min at 4°C. The cell pellet was washed twice with CM medium and seeded into 75-cm² flasks. After seeding, the cell cultures had to be refed at 48 hr with 15 ml of the CM medium supplemented with 20% FBS. Three to five days later, cultures were observed for the appearance of epithelial colonies, and the mixed cell cultures were allowed to grow to confluence. It should be noted that epithelial cell growth was dramatically inhibited by addition of either penicillin, streptomycin, aureomycin or fungizone. Therefore, antibiotics were not added to the culture medium.

**Preparation of epithelial cell cultures.** At confluence density or when cultures reached a diameter of 5 to 9 mm, primary mixed cell cultures were trypsinized in order to remove fibroblasts. The longer the cultures were left in confluence density, the more difficult it became to selectively remove the fibroblast population; 16 hr after the cultures reached confluency proved an optimum time to do this. CM was removed from the mixed cell cultures and the cultures rinsed twice. One milliliter 0.1% trypsin (Worthington Biochemical Corp., lyophilised 9800 BAEE U per mg, lot TL-3BP) in CM was layered over the culture monolayers.

After 90 seconds, the fibroblasts floated off the substratum while the epithelial sheet remained firmly attached to the substratum. The enzymatic action was stopped by the addition of 10% FBS-supplemented CM. Residual fibroblasts were removed by rinsing the flask twice with growth medium. These fibroblasts were seeded in separate culture vessels and subpassaged as previously described (10). The epithelial cultures were fed with 11.5 ml CM medium, 1.5 ml FBS and 3 ml minimum essential vitamin mixture (100x concentrated) [CM plus vitamin supplemented (CM-V), Microbiological Associates, Walkersville, Maryland]. The reduction in supplementation of FBS from 20 to 10% or even 5% diminishes the growth rate of residual fibroblasts while not adversely affecting the growth of the epithelial cells. Trypsinization was repeated 2 to 4 times at 3-day intervals. Epithelial cultures were allowed to grow for at least 2 weeks and were refed every 4 days with CM-V.

**Serial subpassage of epithelial cells.** Subpassage was initiated within 2 to 4 weeks after seeding of the primary cultures. In preparation for subpassage, the CM-V medium was decanted and the epithelial cell sheet rinsed with 10 ml of Mg²⁺-, Ca²⁺-free MEM containing 0.02% 1.50x concentrated vitamin supplemented 10% FBS. Three to five days later, cultures were observed for the appearance of epithelial colonies, and the mixed cell cultures were allowed to grow to confluence. It should be noted that epithelial cell growth was dramatically inhibited by addition of either penicillin, streptomycin, aureomycin or fungizone. Therefore, antibiotics were not added to the culture medium.

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**Fig. 1.** Colonies of epithelial cells after 3 weeks after seeding 500 cells per dish (25 cm²). buffered with formalin and stained with hematoxylin eosin. x14.

**Fig. 2.** Colonies of human fibroblast seeded at 1000 cells per dish (25 cm²) from PDL 2. fixed in phosphate buffered formalin and stained with hematoxylin eosin. x14.
GROWTH OF KERATINOCYTES IN VITRO

TABLE 1

GROWTH CHARACTERISTICS OF FIBROBLAST, EPITHELIAL AND MIXED CELL POPULATIONS IN VITRO

<table>
<thead>
<tr>
<th>Culture Type</th>
<th>Cells seeded (10^3 cm⁻²)</th>
<th>Cell Density at 7 Days (10^3 cm⁻²)</th>
<th>PDL Down After 1 × Passage</th>
<th>Late Span PDL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblast</td>
<td>375,000</td>
<td>1.5 × 10⁶</td>
<td>3</td>
<td>40 ± 3</td>
</tr>
<tr>
<td>Epithelial*</td>
<td>250,000</td>
<td>4 × 10⁷</td>
<td>14</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>Mixed</td>
<td>25,000</td>
<td>2 × 10⁶</td>
<td>3</td>
<td>43 ± 3</td>
</tr>
</tbody>
</table>

* These cell populations exhibit alterations in morphology as they are serially subpassaged.

Preparation of epithelial and fibroblast cultures for microscopy: Cell cultures examined for their growth patterns and morphology in culture were fixed in formalin and stained with hematoxylin. For electron microscopy, epithelial cultures were washed with CM media and exposed to 0.1% collagenase in 10% FBS-supplemented growth medium at 37°C in 4% CO₂-enriched air atmosphere for 4 to 12 hr in order to free the cells or colonies from the substratum. These cell sheets (2 to 50 cm² in area) were removed and fixed in 3% glutaraldehyde in 0.15 M cacodylate buffer, pH 7.4, for 30 min at room temperature or overnight at 4°C. Confluent cultures of fibroblasts were scraped into sheets and fixed in 3% glutaraldehyde. Cell preparations were subsequently post-osmicated in 1% chrome-osmium tetroxide for 1 hr at 4°C. Dehydrated and embedded in Araldite. Thick (1 μm) sections were stained with 1% basic fuchsin in 50% acetone or with aqueous 0.1% toluidine blue for light microscopy. Thin sections (for electron microscopy) were stained with uranyl acetate and lead citrate.

RESULTS

Growth characteristics of epithelial and fibroblast populations. Epithelial cultures, freed of fibroblasts, grew in discrete colonies (Fig. 1). Cell colonies at confluence formed large continuous sheets (~25 cm²) in size. On occasion, the cell sheet would spread up the side of the well or around the neck of the culture flask, a feature never observed with fibroblast cultures. Optimal cell density for seeding of epithelial cultures was found to be 50,000 cells per cm² and a population doubling occurred after 14 days (111). Pure epithelial cultures (PDL 2 to 5) exhibited cellular stratification rather than forming true monolayers; cells were always found to be in contact or intimately attached with the adjacent cells of the patch or...
be detailed in a subsequent paper. Fibroblast populations removed from the mixed foreskin cultures and seeded on a separate substratum exhibited typical fibroblast growth patterns (Fig. 2), growing in definite parallel whorl-like patterns, which were of variable size; cells were not attached to one another. The fibroblast growth characteristics were maintained for PDL 40 (Table 1) when cloned from 100 cells per cm² or at a high density of 5,000 cells per cm². Saturation growth density of subcultures of fibroblasts decreased from 20,000 cells per cm² (PDL 1 to 15) (early phase 2) to 15,000 cells per cm² (PDL 16 to 31) (middle phase 2), and cultures would not reach a confluent state after PDL 32 (Table 1) (late phase 2).

Microscopy of the epithelial cell population. Light microscopic examination of the epithelial colonies demonstrated their stratified nature (PDL 1 to 5). The central region of the colonies was 6 to 8 cells in thickness (Fig. 3), whereas the marginal zone was much thinner and consisted of 3 to 5 cell layers (Fig. 4).

FIG. 4. Transverse section through the marginal region of the epithelial sheet from cultures at PDL 1. Note that the number of cell layers and thickness of this area is less than in Fig. 1. Individual strata can be seen. Stratum basale. B: stratum spinosum. S: stratum granulosum. G: stratum corneum. C: lam Accidite section stained with basic fuchsin. ×700.

Sheet. These growth characteristics were maintained for five PDL after which distinctive changes occurred in the growth pattern that will

Fig. 5. Epithelial cells of the basal (B) and spinousum (S) layers. Note filaments (F) bundles and desmosomes (D). Several mitochondria (M) are also indicated. Stained with uranyl acetate and lead citrate. ×27,000.
Typical keratohyaline granules were not observed in the epidermal cells although present in the native foreskin epidermis. Relatively few mitochondria were present in the epithelial cells of the stratum granulosum and nuclei were uncommon in this layer of the cultured epithelial sheets. Small electron-dense, round-to-oval, membrane-bound granules with electron lucent clefts or zones (Figs. 6–7) provided an additional morphological feature of the granular cells and cells of the stratum spinosum in immediate apposition to the stratum granulosum. It is noteworthy that these small granules concentrated along the plasma membrane on the side of the cell directed toward the stratum corneum. These small granules closely resemble morphologically, and by position, the mucus-coating granules (MCG) described in normal epidermis.

The most superficial layer of the epidermal sheet (Figs. 7, 10) resembled the typical stratum corneum of foreskin epidermis, although this stratum was only one to two cells thick in the culture preparations. The cells were quite flattened and lacked nuclei and cell organelles. Tonofilaments were abundant, oriented parallel to the long axis of the cell and embedded in an amorphous material of low electron density (Fig. 10). The plasma membrane was thickened when compared to the cell membranes of other cells of the epidermal sheet. Modified desmosomes, similar to the modified desmosomes described for normal epidermis, were evident between the cornified cells.

**Fig. 7.** Superficial portion of the epithelial sheet shows a cornified cell (C) and several cells of the stratum granulosum. Secondary lysosomes or lipofuscin granules (L) are evident. In addition, note the small granules (O) concentrating near the plasma membrane. Stained with uranyl acetate and lead citrate. ×21,000.
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Fig. 8. Higher magnification of the secondary lysosomes or lipofuscin granules (L) of granular cell in the stratum granulosum demonstrates their variable internal structure. Desmosomes are shown at D and a portion of an epithelial cell (C) of the stratum corneum. Stained with uranyl acetate and lead citrate. ×27,000.

(Keratinized) cells. Transitional forms between typical granuleal cells and the superficial cornified cells were found.

Morphology of the fibroblast population. Fibroblast cultures derived from the mixed cultures of foreskin appeared quite distinct (Fig. 11) from the cells of the epithelial colonies and sheets. Most of these cells occurred singly. Cells in contact with one another were not attached by desmosomes. Fibroblasts contained both smooth and...
rough endoplasmic reticulum. The rough endoplasmic reticulum was frequently distended and such profiles had few attached ribosomes. Mitochondria were numerous and glycogen was abundant; autophagic vacuoles were present. At higher magnifications, cytoplasmic filaments were evident but were less numerous than in the epithelial cell and did not form discrete bundles as seen in the epithelial cell cultures.

**DISCUSSION**

We have been able to culture and serially sub-pass epithelial cells derived from normal and human foreskin. Pure epithelial cultures free of contaminating fibroblasts were obtained and maintained in typical epithelial-like cultures through five PDL. Epithelial growth patterns were distinctive, and cell colonies, when grown to confluence, formed large sheets several layers in thickness with adjacent cells joined by desmosomal junctions. Self-limiting islands of epithelial cells surrounded by fibroblasts, noted by others using alternative procedures to prepare epithelial cell cultures in vitro (7,9,12-15), were not observed in this study. Cells comprising the epithelial sheets exhibited differentiative changes identical to those occurring in normal epidermis of the intact foreskin. Distinctive cell strata were observed in the epithelial cultures. Epithelial cells or keratinocytes possessed tonofilaments, desmosomal junctions and mucin-coating granules. Thickened cell membranes of the unucleated superficial cornified cells plus modified desmosomes and organised tonofilaments in an amorphous matrix characterised these fully differentiated surface epithelial cells (19). Differentiative changes in each cell strata of the cell sheets were identical with those seen in normal epidermis except that keratohyaline granules (20) were absent in the stratum granulosum; however, secondary lysosomes or lipofuscin granules were a conspicuous feature of the cells of the stratum granulosum of the epithelial cultures. The absence of keratohyaline granules may reflect the cell's inability to synthesise these granules while rapidly

**Fig. 10.** Note the tonofilaments (F) have assumed an orientation along the long axis of the cells of the stratum granulosum. Desmosomes (D) and secondary lysosomes (L) are evident. The plasma membranes of cornified cell (C) is thickened and fine filaments are embedded in an amorphous matrix in this superficial cell of the epithelial sheet. Stained with uranyl acetate and lead citrate. X29,000.
proliferating or because of in vitro culture conditions.

Variable degrees of success have been reported in the propagation of normal human epithelial cells, particularly those from human epidermis or skin. Expiant culture of human epidermis commonly exhibited fibroblastic growth in association with the epithelial outgrowths suggesting that fibroblast interaction was necessary for achieving epithelial propagation and differentiation (161). In the systems described here the presence of fibroblasts does not enhance growth of the epithelial cell population. On the contrary, fibroblasts, when left in mixed cell cultures, overgrow the epithelial cells, thereby inhibiting epithelial growth. After contaminating fibroblasts from mixed primary cultures of human foreskin were removed by selective trypsinization, we were able to subpassage the pure epithelial cell population up to five PDL without morphological alteration. Others (17) were able to subpass normal adult skin through four subpassages with modest differentiation evident in the culture strains.

Growth of human cell populations can be affected by the composition of the fetal bovine serum (15). Traditionally, we characterize (18) the fetal bovine serum prior to use on human cell populations. In addition, we have found that several types of antibiotics inhibited the establishment of proliferating epithelial cell cultures. We have found that the growth characteristics, cell attachments, proliferative characteristics and life span of the cultured epithelial cells from human foreskin were finite and were markedly different from cell cultures arising from a mixed cell population. Fibroblast cultures, derived from infant foreskin subcultures, whose growth to confluency, ceased to grow except at terminal points of the outgrowing patterns; overlapping of cells only occurred at these sites. In contrast, epithelial cells grew in concentric ring patterns and were several cells in thickness.

Preliminary comparison of growth characteristics, as noted in Table 1, indicated that the life spans of epithelial cell populations were different in extent of proliferation from fibroblast PDL.
Moreover, the epithelial cell populations did exhibit characteristic senescent features in vitro. These cell populations, like the fibroblasts, passed through phases 1 and 2 (11) as seen by Karasek and Liu (9). They did not exhibit saturation density-dependent inhibition. The piling up of the epithelial cells into strata (20, 21) may account for the increase in numbers of cells observed in the epithelial cultures. We have found that lesions from adults can produce a ready source of keratinocyte cells that can be grown in vitro using the method described here. These epithelial cell populations also exhibit the growth characteristics associated with tissue in phase 2 and differentiative structures similar in anatomical characteristics to skin epidermis. This differentiative tissue produced in vitro does not require added extrinsic factors, such as epidermal growth factor, pinitary extract, or growth medium, hydrocortisone or the presence of collagen to restrict the proliferation of fibroblasts.

REFERENCES


We acknowledge the expert technical assistance of Donna Parsons and Carol Cunningham in tissue culture and Kathleen Wolken in preparing the samples for electron microscopy.
ESTABLISHMENT OF PROLIFERATING HUMAN EPITHELIAL CELLS IN VITRO FROM CELL SUSPENSIONS OF NEONATAL FORESKIN

Submitted by

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I. INTRODUCTION

The following procedure has been successfully applied to many different human tissues. Tissue samples are obtained from cooperating hospitals. Using the collection techniques described here, we can retain excellent viability from 96 hr up to 5 days (postcollection) depending on the tissue of choice and source of tissues. The use of this procedure permits the establishment of epithelial cell cultures from cell suspensions not requiring explant growth or the addition of extrinsic modulating growth factors. Moreover, epithelial cell colonies can be produced at a low density or high density directly from the cell suspension.

Key words: primary; epithelial cells; call suspensions.

II. MATERIALS

Minimum essential medium (MEM) Eagle with Hank's salts (HBSS) (GIBCO) and 25 mM HEPES; without glutamine and NaHCO₃. To 100 ml of the medium, add 1 ml non-essential amino-acid mixture (10 mM Micro'), 1 ml sodium pyruvate (100 mM solution), 0.1 ml Gentocin (50 mg per ml; Schering'), 1 ml L-glutamine (200 mM'), and titrate with 8.8% NaHCO₃ solution (sterile, carbonate-free) to pH 7.2. Designed complete growth medium (CM).

MEM Eagle without magnesium and calcium① (not supplemented unless mentioned in Procedure section), for suspension (spinner) cultures. Designed spinner medium (SM).

Dulbecco's LoCal medium, Biolabs,② Supplement exactly as CM. Designed LoCal.

Trypsin, lyophilized, No. TL 13 BP Worthington③ (1% solution made up in MEM Eagle HBSS medium)

Essential vitamin mixture. 100X³

Collagenase (CLS). No. 4197.④ Suspend 1 g collagenase in 100 ml MEM Eagle HBSS, pH 7.2; dissolve with magnetic stirrer at 4°C; centrifuge 10,000 × g at 12°C for 10 min and filter through a 0.22-μm filter (No. 7103 Falcon®).

Fetal bovine serum (FBS)⑤ [evaluated for steroid composition (1), unsaturated fatty acid composition (2), mycoplasma contamination, etc. (3), and growth properties on indicator cells (4)]

Stirring bars. Teflon-molded, magnetic, 1-inch long, 1/4-inch diameter. No. 6006 Balco®

Culture plates, four wells per plate. 25-cm². No. 3004® or No. FB-4-TC Linbro®

Scalpels, disposable, steril. No. 32 390-0222 AHS®

Tissue culture flasks: 75-cm², No. 3024® or No. 5373®; or 25-cm², No. 25100 Corning®

Plastic pipettes, measuring (Mohr, plugged: 5-ml No. 7532: 10-ml No. 7548®

Centrifuge tubes, conical, plastic. 15-ml. No. 3013-000®

Ethylenediamine tetracetate (EDTA), tetrasodium salt⑥

III. PROCEDURE

A. Collection of human tissue

1. Supply the operating room with several 4-oz bottles containing 10 ml CM at pH 7.2 supplemented with 5% FBS and 0.1 ml Gentocin per 100 ml medium. These bottles may be stored at 12°C for many
weeks (in our hands, 3 to 4 weeks, depending on composition of the glass). If there is a drastic change in pH, discard the bottles, i.e. if the pH rises above 7.2 (color shifts from red to purple) or falls below 6.5 (color shifts from red to yellow).

2. Collect tissue on the average of 3 or 4 times a week. We have found that when the tissue is kept in CM plus 5% FBS at 12°C, 95% viability is retained up to 48 hr after collection.

B. Processing of tissue
1. Charge each of four wells of a 28-cm² culture plate with 5 ml CM.
2. Place the tissue from the collection bottles into the first well, swirling the medium to wash the tissue.
3. Transfer the tissue to the second well and wash.
4. In the third well, cut the tissue into three or four segments and wash.
5. Transfer segments to the fourth well and mince with two scalpels into 2-mm pieces.
   a. Swirl the medium to rinse the tissue.
   b. Suck off the medium with a narrow-mouth pipette leaving only the minced tissue.
6. Defrost a 5-ml vial of 1% collagenase and add to the well containing the minced pieces. Have ready a 75-cm² flask containing 15 ml CM supplemented with 20% FBS. Transfer the minced tissue and collagenase to the preincubated flask, thereby diluting collagenase to 0.25%.
7. Incubate the tissue at 37°C in a 4% CO₂ environment overnight (16 hr). For a period of 5 to 7 hr of incubation, use 0.5% collagenase.
8. Transfer the digest into a 15-ml plastic or glass conical centrifuge tube. (Use plastic pipette with a 1.5-mm diameter aperture.)
9. Centrifuge the sample for 7 min at 650 × g at 4°C to 12°C.
10. Resuspend the pellet in 5 to 10 ml CM supplemented with 20% FBS: recentrifuge again as described in step 9.
11. Repeat step 10.
12. Preincubate a 75-cm² flask containing 10 ml CM supplemented with 20% FBS for 30 to 45 min at 37°C in a 4% CO₂ environment.
13. Suspend the pellet obtained from step 11 in 5 ml CM at 20% FBS. Seed one 75-cm² flask or three or four 25-cm² flasks with the cell suspension.
14. Incubate the flasks at 37°C in a 4% CO₂ environment.
15. Two days later, rinse the primary culture with CM and refeed with CM supplemented with 20% FBS.
16. Three to five days postseeding, check for epithelial colonies.
17. Fibroblasts also will be present in these cultures. Three to five days after seeding, when epithelial colonies are well established, selectively trypsinize the cultures to remove the fibroblasts.
   PRECAUTIONARY NOTE: This step is a critical procedure and particular attention must be paid to it. The epithelial islands should be left relatively undisturbed.
18. Decant growth medium from the mix cultures and rinse with 10 ml CM.
19. At this time, remove from the freezer the trypsin prepared as a 1% solution in CM at pH 7.2.
   a. Dilute with CM to 0.1%.
   b. Add 1 ml 0.1% trypsin to the cell sheet.
   c. Incubate at 21°C (room temperature) for approximately 30 sec.
   d. Observe the cell sheet under 10X magnification to determine when fibroblasts lift off the substratum. The epithelial patches will remain attached to the flask.
20. To stop the action of trypsin, add 10 ml CM supplemented with 20% FBS and use this medium to wash the cell sheet to remove fibroblasts.
22. Do not attempt to remove all fibroblasts during this first trypsinization. When the edges of the epithelial patches begin to retract, immediately stop trypsin action. It is better to repeat steps 18-20 the next day than to continue to remove all the fibroblasts at this time.
23. Refeed the flasks containing mainly epithelial colonies with CM supplemented with 10% FBS and 3 ml 100X essential vitamins per 100 ml medium. The lowered FBS supplementation retards the growth of any remaining fibroblasts while the vitamin supplementation encourages epithelial growth.
24. Repeat trypsinization procedures two to four times at approximately 3-day inter-
vals or as necessary to free cultures from fibroblasts.

C. Subpassaging epithelial cells

1. Place epithelial cells for 2 to 3 days on LoCal containing additives and supplemented with 10% FBS and 3 ml 100X essential vitamins per 100 ml of medium. Pretreatment with calcium-deficient medium greatly facilitates the lifting off of the epithelial cells from the substratum during trypsinization.

2. Decant the LoCal medium, rinse the cell sheet with 10 ml SM containing 0.02% EDTA, and incubate at 37°C for 3 to 5 min. During this time epithelial cells will begin to separate along their boundaries but will continue to remain attached to the substratum. An increase in refractoriness along the outer limits of each cell will be noticed.

3. Decant the SM and add 1 ml 0.1% trypsin made up with SM containing 0.02% EDTA.

4. After a few seconds, stop the trypsinization by decanting the SM-EDTA medium and add CM supplemented with 10% FBS and 3 ml 100X essential vitamins per 100 ml medium. Transfer the cell suspension to a 15-ml plastic conical centrifuge tube.

5. Centrifuge at 550 x g for 7 min; decant supernate.

6. Resuspend the pellet with CM supplemented with 10% FBS and 3 ml 100X essential vitamins per 100 ml medium, and seed equally into four 25-cm² flasks, or one 75-cm² flask. We use 5 ml of medium containing the cell suspension. Cells should be seeded at high density.

7. After 10 min, gently swirl dishes or flasks to encourage cells to adhere to each other.

8. Return the cultures to the incubator and do not disturb for 3 to 4 days.

9. After 3 to 4 days, examine the cultures for growth of epithelial colonies.

DISCUSSION

There are several critical steps in the procedure that will mean the difference between success and failure. During the initial phase of collection of the tissue, make sure that the pathologist or surgeon does not place the samples into phosphate buffered saline (PBS) or physiological saline, such as Kreb's solution. The carrier medium defined here will keep the tissue viable up to 4 or 5 days at a 95% efficiency. Do not use penicillin-streptomycin, nystatin, mycostatin, amphotericin B, tetracyclines, etc.; they will kill the cells.

All plastic ware, i.e., petri dishes and 75-cm² flasks, should be kept in a constant environmental room at 72°C at a relative humidity (R.H.) of 75%. The flasks under these conditions will remain in acceptable condition for 2% years. The single well and multiwell dishes can be used for up to 6 months when stored under these conditions. All pipettes used to pipette cells should have an aperture of 1.5-mm diameter. The FBS must be evaluated before use (1). Collagenase and purified trypsin prepared specifically for tissue culture applications should be screened for mycoplasma (2). Photographs of vertical stratification of epithelial patches and tables of growth kinetics are presented in Milo, Ackerman and Noyes (4).

V. REFERENCES


FELINE SARCOMA VIRUS INDUCED IN VITRO PROGRESSION FROM PREMALIGNANT TO NEOPLASTIC TRANSFORMATION OF HUMAN DIPLOID CELLS

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Running Title: Neoplastic Transformation of Human Cells
SUMMARY

Human diploid cells morphologically transformed by feline sarcoma virus were serially propagated under selective cell culture conditions. When injected into nude mice prior to passage in soft agar (0.35%), morphologically transformed cells did not produce tumors. However, when propagated under selective cell culture conditions, transformed cells grew in soft agar and, when injected subcutaneously into the subcapsular region of the nu/nu mice, produced neoplastic nodules histopathologically interpreted as fibromas. Karyological examination of cell populations grown out from the tumors confirmed that the tumors were composed of human cells. Examination of electron micrographs of the excised tumor tissue revealed the presence of budding virus particles. Tumor cells isolated from nude mice and morphologically transformed cells both contained the feline oncornavirus-associated cell membrane antigen. It was concluded that expression of feline oncornavirus-associated cell membrane antigen is associated with an early stage of feline retrovirus-induced carcinogenesis, namely focus formation. In addition, it was shown that FeLV-FeSV can induce morphological transformation in human cells in vitro and that there is a requirement for the cells to passage through soft agar before subsequent tumor formation (neoplastic transformation) can be demonstrated.

Key words: Feline sarcoma virus, neoplastic transformation, human diploid cells.
INTRODUCTION

Feline retroviruses have been reported to morphologically transform cells of numerous animal species including hamsters (1-4), cats (5-7), dogs (2,4,8,9), pigs (2,5), sheep (10), monkeys (11), and humans (4,7,9,10,12,13,14,15). However, rat and mouse cells are refractory (16) along with WI-38 cells (17) to feline retrovirus transformation and thus there are no reports about neoplastic transformation, i.e. ability to produce tumors with the in vitro transformed cells in an appropriate animal host.

Although the role of feline retrovirus in spontaneous neoplastic diseases of various heterologous animal hosts is not known, the feline sarcoma virus (FeLV/FeSV) induces fibrosarcomas in cats (5,6,18) and other species. The oncogenic properties of FeLV/FeSV in humans are not known.

The objectives of this study were to determine the optimal in vitro conditions of transformation of human diploid cells by FeLV/FeSV and the oncogenic potential of these transformed cells in nu/nu mice.
MATERIALS AND METHODS

Primary NFS Cultures. Primary human cell cultures (NFS) established from foreskin tissue as previously described (19), were maintained on Eagle's Minimal Essential Medium (MEM)-Hank's buffered salt solution (HBSS)-25 mM Hepes buffer at pH 7.2 (Gibco, Grand Island, N.U.), 1 mM sodium pyruvate, 2 mM glutamine, 50 μg gentamycin per ml (Schering Diagnostics, Port Reading, N.J.), 0.2% sodium bicarbonate, and 10% fetal bovine serum (FBS) (Biofluids, Inc., Rockville, MD).

Preparation of Stocks of Snyder-Theilen FeSV. Stocks of ST-FeSV were prepared as previously described (20). Feline embryo cells at 50-70% confluency were treated with DEAE-Dextran (40 μg/ml) in L15 medium (Gibco, Grand Island, N.Y.) at room temperature for 20 min. Following removal of the DEAE-Dextran, a virus inoculum in L15 medium supplemented with 5% FBS was added to the cell sheet for 2 hours. The cell sheet was then refed with L15 medium + 15% FBS and incubated for 7-10 days at 37°C in a 4% CO₂ enriched atmosphere. The cells were harvested by scraping and subjected to two cycles of rapid freezing in dry ice/95% ethanol and thawing in a 37°C water bath. The cell suspensions were centrifuged at 350 x g for 10 min; the supernatant solution was filtered through a 0.45 μm Millipore filter and stored in 0.5 ml aliquots at -70°C.

Selection of "Pure" Populations of FeSV-infected Human Cells. Preconfluent cell populations at population doubling (PDL) 4-14 (1) were seeded at 0.5-1.0 X 10⁶ cells per sq cm in MEM + 10% FBS. After each split at a 1:4 split ratio, the PDL were increased by 2. After 24 hours, the cells were inoculated with 1.250 dilution of FeSV which had a titer of 6.1 X 10⁶ ffu per ml. The protocol for inoculation was identical to that used on the human diploid cell cultures except that MEM growth medium was used in place of L15. Ten days later, the cultures, which contained 10 foci per sq cm for a
20 sq cm plate, were passaged 1:4. Those areas in the confluent culture containing hyperrefractile cells that stained densely with hematoxylin were identified as foci (12). The cultures attained confluency in five days and were serially passaged thereafter 1:10 until "pure" populations were attained that contain 100% Feline Oncornavirus Cell Membrane Antigen (FOCMA)-positive cell populations. These infected cell populations required 8-9 days to reach saturation density.

**Effects of Different Growth Media on Proliferation of FeSV-infected Cells.**

Cells were seeded at 1:10 dilutions into different growth media supplemented with 10% FBS. Cell proliferation was then monitored in either McCoy's 5A, MEM-Mg\(^{2+}\), MEM (Gibco, Grand Island, N.Y.) or EBM LoCal (Biolabs, Northbrook, Ill.). McCoy's 5A medium was used previously for the growth of feline leukemia virus transformed (FL-74) cells in suspension culture (20); MEM-Ca\(^{2+}\) - Mg\(^{2+}\) and EBM LoCal were selected because these elements have previously been found to alter Adeno-12-induced focus formation of hamster embryo cells (21), and to alter susceptibility and refractoriness of Yaba tumor pox virus-induced focus formation (22).

**Release of Infectious Virus from FeSV-infected Human Cells.** Pure populations of FeSV-infected human cells growing on either EBM LoCal, McCoy's 5A, or MEM media were assayed for the release of infectious virus. Twenty-four hours after seeding at PDL 20, or as the cells stopped proliferating, aliquots of the supernatants were removed and filtered through a 0.22 μm Swinnex Millipore filter, then diluted and used as inoculum. Ten days later, the infected plates were fixed in 10% formalin and stained with hematoxylin and eosin. Finally, the foci were enumerated. These foci contained cells that were morphologically distinct from the normal cells (see above) and are hereafter referred to as morphologically transformed cells.
Passage of Morphologically Transformed Cells Through Soft Agar. Soft agar was used as a suspension medium for the growth of morphologically FeSV transformed cells. A 2% agar base, RPMI 1629 medium (Gibco, Grand Island, N.Y.) was supplemented with 20% FBS. Transformed cells were harvested from the supernatant and resuspended in EBM LoCal medium + 20% FBS, 1% essential amino acids, 1% essential vitamins, 0.35% agar, 1 mM sodium pyruvate, 2 mM glutamine, 0.2% sodium bicarbonate and 50 μg/ml gentamycin. Two ml of this cell suspension were seeded at 1-2 X 10⁵ cells/ml over the agar base plates. These media were used because the morphologically transformed cells grow more optimally in these media. We have tried PHMI 1640, MEM, BME, etc., with a lesser extent of success.

FOCMA Detection. An indirect immunofluorescence test for FOCMA (23) was performed on the morphologically transformed cells. Proliferating transformed cells from either monolayers or soft agar were harvested by centrifugation at 650 x g for 7 min at a cell density of 0.5-1.0 X 10⁶. The reference primary reagent (cat serum) used in this study was from a FOCMA antibody positive cat that was persistently viremic. This agent was shown to be specific for FOCMA, since absorption of the serum with intact and ether-disrupted FeLV (10⁶ purified particles per ml of serum) did not decrease antibody titers (24). In addition, this reagent produced membrane fluorescence on FeSV-infected human neonatal foreskin cells, but not on uninfected human foreskin cells (unpublished data).

Histopathology and Electron Microscopy. Tumors of FeSV-infected cells and FeSV-infected cell populations prepared from boluses growing in soft agar were prepared for histopathology and electron microscopy.

Athymic nude (nu/nu) mice, backcrossed 5 or 10 times which were obtained from Sprague-Dawley, Madison, Wisconsin were selected for evaluation of the neoplastic
potential of FeSV-transformed human cells. Preconfluent cultures of FeSV-transformed cells were prepared for injection by scraping with a rubber policeman and were pelleted by centrifugation at 340 x g for 7 min. The pellet was resuspended in fresh MEM and recentrifuged. After resuspension in MEM - 0.5% agar, 0.53-1.0 X 10^7 cells were injected subcutaneously into athymic nude (nu/nu) mice which had been irradiated 3-4 days previously with 450 rads ^137Cs γ-rays. The nodules which developed at the site of inoculation were excised after six weeks' growth, fixed in 3% glutaraldehyde-0.1 M cacodylate buffer at pH 7.4, then prepared for histopathology and electron microscopy. In addition, FeSV-infected proliferating populations isolated from boluses obtained from soft agar were scraped from the substratum of the flasks, pelleted by centrifugation at 650 x g for 7 min, and fixed in 3% glutaraldehyde - 0.1 M cacodylate buffer at pH 7.4 for examination under an electron microscope.

Karotype Analysis of Excised Tumors. Tumors from 0.8 - 1.2 cm in length were identified at the site of injection after 6 weeks. These tumors were surgically removed from the nude mice and cell suspensions were made as described elsewhere (19). Hyperimmune antiserum prepared against nude mouse skin cells was added to the culture of 500,000 tumor cells in a 75 sq cm flask at 0.6 ml per 15 ml of growth medium. The medium containing the antiserum was renewed every 24 hours. Seventy-two hours later, the proliferating cells were refed with 5 μg/ml of colcemide (Gibco, Grand Island, N.Y.) in 10 ml of growth medium and incubated for 3 hours at 37°C. The medium was decanted after 3 hours and the cell monolayer was rinsed with warm PBS-Ca^{2+} - Mg^{2+}. Following their removal with 10 ml of trypsin-verseine solution (40:1), the cells were recovered by centrifugation at 650 x g. The cell pellet was fixed in cold methanol-glacial acetic acid (3:1). A suspension was dropped onto a glass slide, dried and stained with prefiltered 3% Giemsa solution.
RESULTS

Selection of Pure Populations of FeSV-infected Cells. At five to 10 PDL after starting selective subculturing of the virus-infected human cells was started, it became impossible to distinguish individual foci in the culture because of the increased number of infected cells in the population. Many hyperrefractile round FeSV-infected cells were released into the growth medium. Populations of 1-2 x 10^6 cells/ml were harvested from this "breeder" culture. Two methods of harvesting these free-floating cells resulted in the selection of two cell types. Centrifugation and resuspension in fresh MEM + 10% FBS gave rise to cells which attached to the substratum and exhibited a variety of cellular morphologies (Fig. 1). Direct transfer of the old media and cells into a flask produced a seeding suspension; some of these cells attached to the substratum, while others began to grow into large boluses in suspension which varied in size and contained from 25-200 cells (Fig. 2). Both harvesting methods gave rise to cultures containing pleomorphic cellular and colony morphology.

Growth Characteristics in Different Culture Media. We have tried many recipes for media. Table 1 lists their ability to support the growth of the transformed cells. MEM-Mg^{2+} + 10% FBS, McCoy's 5A + 10% FBS, and McCoy's 5A supplemented with 5 μg/ml spermidine + 10% FBS supported cell growth for less than 3 PDL. McCoy's 5A - 5 μg/ml uridine - 10% FBS supported growth for 3-5 PDL.

FeSV-infected cells subpassaged 1:10 into EBM LoCal medium plus 10% FBS proliferated for 6 PDL; however, further subpassaging resulted in cell lysis. Cultures that were subpassaged on MEM-Hepes died after 42-46 PDL.

Release of Infectious Virus. The supernatants were individually assayed for infectious virus (see Materials and Methods) after 20 PDL or when the cells ceased proliferating and lysed.
The data in Table 2 indicate that only MEM and EBM LoCal supported the production and release of infectious virus. There were 350 times more ffu released from cells grown on MEM than from cultures grown on LoCal. This suggests that virus production and release, like cell proliferation, is dependent on the specific cell culture medium used. Calcium has also been shown to alter virus-induced cell morphology and to produce biochemical changes in other virus-cell systems (15, 25). Cell populations grown in McCoy's 5A supplemented growth medium died after a short time. There was no release of detectable infectious units.

**Growth in Soft Agar.** Single cell clones were obtained by cloning single cell suspensions in agar to evaluate the infected cells for their ability to grow in soft agar. Free-floating cells were seeded at 1-2 X 10^3 cells/ml into soft agar after 16-20 PDL and round compact colonies observed 10-14 days later containing 50-100 cells per bolus (Fig. 3). The frequency of bolus formation was 2.5 to 5 X 10^-4.

**FOCMA Expression on FeSV-infected Cells.** Randomly proliferating cells were assayed for the presence of FOCMA by indirect immunofluorescence (26). Figure 4 (A) is a light micrograph of several infected cells. Figure 4 (B) illustrates the fluorescent pattern of FOCMA on the same cells. The pattern is typically patchy. We observed a similar pattern of FOCMA fluorescence by FL-74 cells grown in spinner flasks (20). To date, all transformed cells treated with fluorescein isothiocyanate tag control cat serum have been negative.

**Growth of FeSV-transformed Human Cells in Nude Mice.** To evaluate the neoplastic potential of these FeSV-transformed proliferating human cells, 0.5-1.0 X 10^7 cells were injected into nude mice. Twenty-four hours later, the bleb at the injection site regressed. After 5-30 days, palpable nodules were evident. They increased in size.
to 0.3 - 1.4 cm over a four-week period at the end of which the nodules were excised and prepared for histopathology.

**Histopathology of Tumors from Nude Mice.** Histologically, the nodules from both mice were well encapsulated, sharply demarcated cellular masses (Fig. 5). The neoplastic cells were ovoid to spindle-shaped and contained a single vesiculated nucleus usually with a prominent nucleolus. The cytoplasmic margins were frequently indistinct and some cells appeared to form syncytia. An application of the Massons trichrome stain showed the eosinophilic fibrillar intercellular material to be collagen. A basophilic (hematoxylin) mucin-like intercellular material was admixed with the collagen fibers and stained with Alcian blue. This revealed the presence of acid mucopolysaccharides (Fig. 6). Mitotic figures were rare, the mass was well vascularized and the neoplastic cells were located immediately adjacent to the vessels. The mass was interpreted to be a fibroma.

Under electron microscopic examination, cells in culture and cells from the nodules removed from mice were both found to contain virus-like particles (Fig. 7).

**Distribution of Chromosome Number in Tumor Material.** Tumors excised from nude mice were grown in vitro as described previously. In no case of the metaphase spreads from 5 different tumors evaluated was there significant deviation from the diploid number of chromosomes.
DISCUSSION

Replication of FeLV-FeSV in human cells and subsequent focus formation were demonstrated in this study, confirming previous reports (9,10,15,26,27,28,29). The data obtained in this study, however, suggest that focus formation (i.e. morphological transformation) (2) represents only a transitional stage in the neoplastic transformation process. It is interesting to note that Azocar and Essex (17) did not observe morphologically transformed cells when WI-38 were infected with FeSV. However, if we add fungizone or penicillin-streptomycin to the cultures much in the same manner they did, no foci were observed. This study showed that the separation of morphologically transformed from nontransformed normal cells and growth and passage in soft agar were prerequisites for the demonstration of neoplastic properties by the transformed cells. The separation was accomplished by culturing and passaging the cells in a low-calcium supplement growth medium for 2 PDL to 5 PDL in MEM-Hepes EBM-LoCal medium. The resultant transformed cell populations grew in suspension, while populations that contained normal-appearing cells did not. Selective culturing in suspension of these cells in EBM-LoCal medium followed by subculturing in MEM 5 PDL later resulted in the cells reattaching to the substratum. It was necessary to serially subpassage these cells for an additional 16-20 PDL in 1 X MEM-Hepes growth medium before they would grow in soft agar. The morphologically transformed cells, when passaged through soft agar, formed colonies of 50-100 cells per bolus at a frequency of $2.5 \times 10^{-4}$ per 25 sq cm. These cells, isolated from soft agar and grown in a selective medium, were injected into 6-week-old nude mice to evaluate the oncogenic potential. Nutritional requirements and time in culture appear to determine growth and expression of the neoplastically transformed cells. Selective nutritional requirements have also been shown for other FeSV-transformed cell systems such as
transformed feline producer cells which grow optimally on McCoy's 5A (22) or Ad-12-transformed hamster cells which require a low-Ca\(^{2+}\) growth medium (21). The transformed human cells that also grew on EBM-LoCal medium did not grow on MEM minus Mg\(^{2+}\) - Ca\(^{2+}\).

Tumors produced in the nude mice (0.3 - 1.4 cm in size) were found to contain collagen and were interpreted histopathologically to be fibromas, not fibrosarcomas (23). Removal of the tumor was followed by growth of the cells in culture; subsequent karyological examination indicated that the tumor cells were of human origin. Examination of electron micrographs of excised tumors and proliferating cells from in vitro populations of morphologically transformed cells, and examination of cells passaged through agar, revealed that both types of cells were shedding virus particles. Assay of the morphologically transformed cell populations (30) before and after growth in soft agar revealed that FOCMA was present at both stages in the transformation sequence. These results suggest that FOCMA expression is associated with the early events in the transformation process. There appears to be a program or staging process that must occur in FeVL/FeSV-transformed human cells before they will produce tumors when injected into a suitable host. The results reported here appear to be similar to the multistage process of chemical carcinogen-induced transformation (23). FOCMA and virus expression appear to be associated with early stages of morphological transformation, while growth in soft agar is associated with a later transitional stage. A high correlation exists between growth in soft agar and tumor formation. It was noted that while virus-infected cells would not produce tumors prior to their growth in soft agar, after they were passaged through the soft agar they would produce tumors. Again, this suggests that growth in agar acted as a selection process to permit the proliferation of cells that can produce tumors when inoculated into a
suitable host. Therefore, we have shown that FeSV neoplastically transformed cells are capable of producing tumors in nude mice.

The release of the infectious virus from human cells that form tumors or grow in soft agar raises the question of possible horizontal transmission. FeLV and FeLV/FeSV have been shown to be horizontally transmissible among cats (30) and FeLV, under laboratory conditions, was horizontally transmissible to dogs (15). A serological survey indicated that no antibody to FeLV occurred in hundreds of individuals exposed to viremic cats (12,31). However, Jacquemin et al. (32) reported that purified human IgG from patients with chronic myelogenous leukemia specifically neutralized reverse transcriptase isolated from FeLV.
ACKNOWLEDGEMENTS

We would like to thank Mr. Pat Adams for expert technical assistance. This work was supported in part by NIH-NCI RO1-25907, NO1-CP-3571 and CPVO 103563, and Air Force F49620-77-C-110.
REFERENCES


Growth of FeSV-Transformed Cells in Different Growth Media

Table 1

<table>
<thead>
<tr>
<th>Growth Medium</th>
<th>PDL</th>
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<tbody>
<tr>
<td>(1) MEM + 25 mM Hepes</td>
<td>46</td>
</tr>
<tr>
<td>(2) MEM - Mg(^{2+})Ca(^{2+})</td>
<td>&lt;3</td>
</tr>
<tr>
<td>(3) McCoy's SA</td>
<td>0</td>
</tr>
<tr>
<td>(4) McCoy's SA + 5 μg/ml spermidine</td>
<td>&lt;2</td>
</tr>
<tr>
<td>(5) McCoy's SA + 5 μg/ml uridine</td>
<td>4</td>
</tr>
<tr>
<td>(6) EBM LoCal</td>
<td>7</td>
</tr>
</tbody>
</table>
Table I

The proliferative characteristics of a pure FeSV-transformed human cell population were measured as PDL on different types of growth media. Other experiments on other populations for PDL 1 through 6, not reported here, supported these data. In most cases the mean PDL did not exceed one sigma S.D. for values reported here. PDL as referenced in the text is: 1 serial subpassage at 1:2 split ratio at 95% absolute plating efficiency. One population doubling (PDL) is that cell population that was serially passaged 1:4 at confluency. If the cells were serially passaged at 1:4 they had proceeded through PDL.
Measurement of Focus Forming Units on Appropriate Monolayer Cultures.

Table 2

<table>
<thead>
<tr>
<th>Growth Medium</th>
<th>ffu/75 cm² flask*</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) MEM + 25 mM Hepes</td>
<td>$1.9 \times 10^7 \pm 1.4 \times 10^5$</td>
</tr>
<tr>
<td>(2) MEM-Mg²⁺-Ca²⁺‡‡</td>
<td>---</td>
</tr>
<tr>
<td>(3) McCoy's 5A ‡‡</td>
<td>---</td>
</tr>
<tr>
<td>(4) McCoy's 5A + 5 μg/ml spermidine ‡‡</td>
<td>---</td>
</tr>
<tr>
<td>(5) McCoy's 5A + 5 μg/ml uridine ‡‡</td>
<td>---</td>
</tr>
<tr>
<td>(6) EBM LoCal</td>
<td>$5.5 \times 10^3 \pm 1.1 \times 10^3$</td>
</tr>
</tbody>
</table>

* Dashes (---) mean that ffu were not detected when supernatant solutions were assayed on appropriate human monolayer cultures (19) (Morphological Transformation) after the cell populations ceased to proliferate and lysed (Table 1).
Table 2

These figures show the results of a focus-forming assay of FeSV-transformed cells cultured in different growth media. NFS cells were originally infected with a 1:250 dilution of FeSV from a pool with a titer of $6.1 \times 10^4$ ffu/ml. The virus assay period was determined to be at least 20 PDL at 1:10 split ratios after virus infection for cells grown on MEM-Hepes medium. This time period was selected in order to ensure that only pure populations of virus-transformed cells were assayed. Focus-forming units/75 sq cm flask were determined for $1.5-2.0 \times 10^6$ cells per experiment. These data represent the results for n of 4 for MEM at PDL-20 and n of 3 for LoCal at PDL 7. They are presented here as mean values ± 1 sigma standard deviation.

* Cells cultured in these media exhibited limited proliferative capability and ceased proliferating prior to the virus assay period.
Modality of Human Chromosomes of Cells Prepared from Tumor Cells.

Table 3

<table>
<thead>
<tr>
<th>PDL*</th>
<th>Model No.</th>
<th>Range</th>
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<tbody>
<tr>
<td>10</td>
<td>45</td>
<td>43-47</td>
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<td>46</td>
<td>45-47</td>
</tr>
<tr>
<td>7</td>
<td>46</td>
<td>45-48</td>
</tr>
<tr>
<td>6</td>
<td>45</td>
<td>44-46</td>
</tr>
</tbody>
</table>

* These PDL represent proliferation of population of FOCMA positive cells seeded from the tumor. The range represents the distribution of chromosomes seen on a single slide.
Table 3

The data presented here represents our attempt to count the numbers of human chromosomes found in the cells that produced tumors in the nude mice. The tumors were removed seeded in vitro and at subsequent PDL evaluated.
Figure 1

This photograph shows proliferating FeSV-transformed human cells. A supernatant cell suspension from a culture at PDL 17 containing 70-90% transformed cells was harvested by centrifugation at 650 x g for 7 min and the cell pellet was resuspended and seeded in MEM + 10% FBS. Other nonattached cells remain rounded up and floating in the medium. Magnification X40.
Figure 2

A cell suspension recovered without centrifugation from the overlying growth medium of FeSV-transformed human diploid cells, PDL 16, was seeded directly into a flask without replenishing the growth media. Ten days later actively dividing boluses of cells were observed. Magnification X32.
Figure 3

A suspension of free-floating viable cells in the overlying growth medium from a FeSV-transformed culture at PDL-12 was harvested by centrifugation at 650 x g for 7 min. Cell populations at 1-2 x 10^5 cells/ml were seeded into 0.5% soft agar containing LoCal growth medium. Ten days after seeding, colonies of cells were observed. Magnification X82.
Figure 4

Proliferating FeSV-transformed cells, 0.5-1.0 × 10^6, were harvested by centrifugation at 650 x g and incubated with FOCMA antibody reference cat serum, obtained from an animal with a regressing sarcoma, for 30 min. Cells were washed free of unbound cat globulins with three consecutive rinses of Hank's balanced salt solution and incubated with a 1:20 dilution of goat anti-cat gamma-globulin (Sylvania, Inc., Milburn, N.J.) which had been conjugated to fluorescein isothiocyanate. (A) Light micrograph of FeSV-transformed cells, (B) UV-fluorescence micrograph of the same field. Magnification X250.
Figure 5

A well-encapsulated subcutaneous nodule in an np/np mouse. The cells are individually aligned (arrow) or arranged in syncytial sheets (s). Magnification X10.
Electron micrograph of cell from subcutaneous nodule in \textit{nu/nu} mouse. The interstitium contains flocculent electron-dense material (arrowhead) and numerous fibrils (arrow) containing the characteristic 640 Å banding of collagen. Magnification X45,000.
Figure 7

Virus particles (arrows) budding from cell membrane of fibroblast in culture prior to injection into ημ/ημ mouse. Magnification X28,000.
Ultraviolet Radiation Induced Neoplastic Transformation of Normal Human Cells, In Vitro

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Abbreviations: UV, ultraviolet 254 nm radiation; E.D. 50, effective dosage 50%; PDL, population doubling; CM, complete growth medium; FBS, fetal bovine serum; PBS, phosphate buffered saline at pH 6.8; J.m^-2.sec^-1, Joules per meter^2 per second^-1; S phase, scheduled DNA synthesis; RCE, relative colony forming efficiency.
SUMMARY

Human foreskin cell cultures in S phase of the cell cycle were exposed to UV radiation at a dose cytotoxic to 50% or less of the cells, in the presence of insulin. Cell populations treated at a dose less than E.D. 50, when selectively subpassaged in a high amino acid supplemented CM after 20 PDL, were able to grow in soft agar. Cell populations 5 PDL after treatment exhibited altered lectin agglutination patterns but would not grow in soft agar. UV-treated cell populations also grew in a reduced serum concentration and at 41°C. These indices, along with abnormal colony morphology, appeared to be associated with early events in the expression phase of the transformed phenotype. After 20 PDL in the selective CM we observed a frequency of 20 colonies in $10^5$ cells seeded in soft agar. The cell populations derived from these colonies, when propagated and injected into the nude mice, formed myxofibromas at the injection sites rather than the type of tumor (fibrosarcoma) previously described for chemical carcinogen-induced neoplasms.
INTRODUCTION

Carcinogen-induced transformation of human cells in vitro by chemicals or irradiation has been difficult. Refractoriness of human cells to in vitro transformation is not unique. Recently, however, neoplastic transformation of human cells by chemicals (1) and viruses (2) has been achieved. In addition, Sutherland (3) using U.V. treatment of human cells damaged the DNA and transformed the cell populations to an anchorage independent state of growth. It was necessary to use multiple treatments at subtoxic doses of U.V. to damage the DNA and induce transformation. Other studies with refractory human cells have indicated that carcinogens can induce unscheduled DNA synthesis (4) or repair synthesis (5). However, these reports do not attempt to correlate damage to DNA with expression of carcinogenesis. Instead, Heflich (4) correlated removal of chemical carcinogen and induced damage to DNA with cytotoxicity (4). In this report, we have expanded on our preliminary report of U.V. induced neoplastic transformation of human cells (7) and present data here on the reproducibility of the process. We also present data on the interrelationship between anchorage-independent growth and tumor growth as the normal cells pass through a retrodifferentiated sequence from induction to neoplasia.
MATERIALS AND METHODS

We have found that in order to observe reproducible transformation of human cells exposed to U.V. it was necessary to first use low passage cell populations (PDL 1-3) and, second, to complete a cell survival curve prior to the selection of an appropriate treatment dose.

Cell Cultures

Primary cell cultures were obtained from neonatal foreskins (NFS) as previously described (8) and maintained on CM, viz., minimum essential medium (MEM; 25 mM HEPES, GIBCO, Grand Island, New York) at pH 7.2 supplemented with 10% FBS, Gibco, Kankakee, Illinois; 1 mM sodium pyruvate, 2 mM glutamine, 0.2% sodium bicarbonate and 50 ug/ml Gentomycin, in an atmosphere of 4.0% CO₂-enriched air at 37°C.

Survival Studies of Irradiated Cells.

Immediately after UV treatment and/or 40 h later, cell survival was measured by either dye exclusion (9), colony forming ability (10), or incorporation of ³H-thymidine into cellular DNA (9). After seeding, cultures were irradiated with a 15W Germicidal Electric lamp (15GT8) at a fluence rate of 1.2 J. m⁻². sec⁻¹. The fluence rate was measured by a Blak-Ray UV meter (UV Products, International, San Gabriel, Calif.). If wished to measure colony forming capability of the treated cell population, one thousand cells were seeded in 25 cm² wells (Falcon Plastics, Oxnard, Calif.) and fed with CM supplemented with 20% FBS. These cultures were incubated at 37°C in a 4% CO₂ enriched air atmosphere for 9 days, fixed in phosphate -3% formalin stained with Hematoxylin-Eosin, and enumerated under 2X. If the effect of the treatment on cell survival was to be measured by dye exclusion, approximately 5000 cells -cm⁻² in a 25 cm² dish were trypsinized at the conclusion of the UV treatment and counted in a cytometer (9). Inhibition of the incorporation of (³H)-CH₃-thymidine (S.A. 34,0
Cl/m mole) into the DNA of UV treated cells was measured by sampling the cell population from 0-48 hrs following initiation of JV treatment (8). At the sample times 1 hr intervals) three coverslip cultures were removed, fixed in 3:1 methanol:acetic acid solution, acid washed in 1.0N HCl and air dried. Two coverslips were transferred to scintillation vials and incubated overnight with 0.5 ml of NCS tissue solubilizer, neutralized with 1N NaOH. Ten ml of Insta-Gel (Beecham Inst., Palo Alto, Calif.) was added to each vial and the radioactivity counted in a Beckman scintillation counter.

Transformation

Once the cell survival studies were completed, we observed that all cell populations, when treated at a survival dose of 50%, optimize the formation of transformants.

The scheme of induction and selection of the transformed cells is presented along with the chronology of the events as they occurred (Fig. 1).

To maximize the number of UV-induced lesions going through scheduled DNA synthesis, the cultures were irradiated at the beginning of S phase (1). Preconfluent, logarithmically growing cultures at low (3-8) PDL were synchronized by placing them at a density of 5,000 cells-cm⁻² into Dulbecco’s Modified Eagle’s Medium (Biolabs, Northbrook Illinois) at pH 7.2, supplemented with 10% dialyzed FBS but lacking arginine and glutamine. After 24 h, the amino acid deficient medium was replaced with CM to which 0.5 U/ml of insulin had been added. Ten h later, when the cell population was entering S phase of the cell cycle (1), the growth medium was removed, and the cultures were washed twice with Dulbecco’s phosphate buffered saline, irradiated with 5.0 J m⁻² UV and refed with CM containing 0.5 U/ml insulin. Upon completion of the treatment the cells were allowed to proceed through S phase, which required 8.2 h, and enter mitosis. They were then split 1:2, then 1:10, into MEM supplemented with 10% FBS plus 8X nonessential amino acids (8X growth medium). The 8X growth medium inhibited
growth of cells that exhibited a normal phenotype (5), while allowing proliferation of transformed cells.

The transformed cell population at this time was identified as entering the early stage of the carcinogenesis process. During this stage of expression it is imperative that no part of culture remain in a saturation density growth state for more than 16 hrs prior to subpassaging the culture.

**EARLY**

Growth of cells at 41°C and in medium with reduced serum concentration.

Cell populations, following treatment with UV, were seeded (5000 cm⁻²) in CM and serially subpassaged at 1:4 on a 4-day schedule and maintained at 41°C in a 4% CO₂-enriched air environment. Cell populations from companion cultures were seeded at 5000 cells-cm⁻² in CM supplemented with 1% FBS, also in a 4% CO₂-enriched air environment.

**Lectin aglutination**

Treated and control populations at a 70-90% confluent density of proliferating cells were removed from the substratum of the flask by the action of 0.05% trypsin (3). Following centrifugation at 630 x g, the pellet was resuspended in PBS at pH 6.8 and recentrifuged twice. The final cell suspension was left on wet ice at a cell density of 10⁶ cells ml⁻¹. Wheat germ agglutinin prepared in PBS was added to the wells of microtiter plates (0.025 ml). To this, 0.025 ml of a suspension of 180,000 cells in PBS was added to each well and incubated at 21°C for 10 minutes. The wells were then examined immediately.

**TRANSITIONAL**

**Soft Agar**

After serial passage of the UV treated cells for 20 PDL in 8X amino acid supplemented CM, 50,000 cells were seeded in 2 ml of 0.35% agar supplemented with
modified Dulbecco's Lo-Cal medium (Biolabs, Northbrook, Ill.), overlaid on a 2 ml 2% agar base supplemented with RPMI 1629 growth medium enriched with 20% FBS. Colonies formed after 14-17 days' incubation in a 4% CO₂-enriched air environment at 7°C. These cultures were refed every 7 days with 0.5 ml of Lo-Cal medium-supplemental agar. Since a close correlation has been shown between the growth of chemically transformed human cells in soft agar and their neoplastic potential (1), growth of the UV-treated cells in 6-week-old male athymic nu/nu mice from a BALB/c background (Sprague-Dawley, Madison, Wisconsin) was chosen as a suitable assay system to determine the neoplastic potential of the cultures derived from UV-treated cells.

Growth in nude mouse

Cells were harvested by trypsinization and resuspended in Dulbecco's EBM LoCal (Biolabs, Northbrook, Ill.) supplemented with 20% FBS, 1% essential amino acids and 0.35% agar. Six-week-old nude mice, which had been irradiated 3-4 days previously with 450 rad 137Cs-rays, were injected subcutaneously with 0.5 x 10⁷ UV-treated cells. Nodules became evident at the site of inoculation 14-21 days following injection and continued increasing in size. The blebs formed at the injection site regressed in 48-72 hrs.
RESULTS

Toxic response

In the early stages of our experiments, following the treatment with UV, we measured the toxic effect the treatment had on the UV-insulin treated cells by counting the total number of viable cells at 12 h (Fig. 2) immediately after initiating the UV treatment, or at 40 h after initiating treatment (Fig. 3). In addition, recovery of cell division from the toxic effects of irradiation, monitored by $^3$H-thymidine incorporation into DNA and by cell counts, began at 44-48 h following the completion of the carcinogen treatment at an E.D.50 cytotoxic dose as measured by colony forming capability. The irradiated cultures not exposed to insulin exhibited a typical shoulder on the survival curve followed by a logarithmic decline at doses greater than 10 J.m$^{-2}$. However, cell cultures treated with 0.5 U/ml insulin and then irradiated lacked a shoulder at 10 J.m$^{-2}$, and were biphasic in nature. The toxic effect of increasing UV fluence was more sensitively measured by relative cloning efficiency (RCE) than by the trypan blue dye exclusion technique; it took roughly a 10-fold higher fluence to demonstrate a measured effect (Fig. 2) by dye exclusion than by inhibition of colony formation (Fig. 3). Nevertheless, whichever method we used to measure the response, the survival slopes were similar (Figs. 2,3). The response profiles of insulin-free cultures were similar to those reported by Lehman et al. (11) or Maher et al. (12) for skin fibroblasts from different normal individuals. We noticed that when insulin-treated cells were irradiated at 5 J.m$^{-2}$ (Fig. 3) in S phase there was an increased survival rate of up to 120% over the insulin-free cultures.

Insulin-free non-UV treated synchronized cell populations exhibited an 18% RCE. Randomly proliferating cell populations treated with insulin-UV or UV alone, or synchronized cells treated with UV alone, exhibited a shoulder at fluences of 10 J.m$^{-2}$ (Fig. 3). Insulin treatment appeared to increase sensitization of synchronized cell
populations to UV treatment at fluences of 10 J. m^{-2} up to 20 J. m^{-2}. The insulin-UV-treated cultures at 20 J. m^{-2} exhibited a RCE of 3%, compared to 6% for cells not exposed to insulin. However, the cultures which were exposed to insulin during the 40 h after irradiation did not exhibit a shoulder and the shape of the survival curve was multiphasic.

**Growth characteristics of UV treated cells (Selective Process)**

After 10-15 PDL in the 8X growth medium, the irradiated cultures appeared pleomorphic and contained more than 95% small polygonal cells, many of which were multinucleate and displayed multiple processes as seen by Contrast Interference Nomarski microscopy. Cells passaged from these cultures proliferated to saturation densities of 4-6 x 10^6 cells/75 cm^2 flask in 8X growth medium, whereas untreated NFS cells only reached 1.2-1.5 x 10^6 cells/75 cm^2 flask. Treated cells exhibited a loss of contact inhibition, a tendency to pile up in culture, and a pattern of irregular criss-cross growth. None of these altered characteristics were observed in control cultures. These cell populations were capable of subpassage to 120 PDL while the controls phased out after passage to 40 ± 5 PDL.

To examine the cultures for growth at elevated temperatures, populations were subpassaged 1:4 and incubated at 37°C in a humidified 4% CO_2 atmosphere to allow cell attachment to the substratum. The temperature was then raised to 41°C. As early as 10 PDL after irradiation, cell cultures derived from UV-treated populations actively proliferated at 41°C for 72 h, at which time they were 60-70% confluent. Control cells detached from the surface in less than 24 hrs.

**Growth in reduced serum**

Many transformed mammalian cells have been reported to grow at reduced serum concentrations (13,14,15). We examined our control and UV-treated human cell populations for this characteristic after 10 PDL by subpassaging into MEM
Supplemented with 1% FBS. Table 1 represents the growth characteristics of these cultures at reduced serum levels. Control NFS cells ceased proliferating after 3 PDL. After an initially slow growth rate, the UV-treated cultures appeared to adjust to the low serum concentration, as shown by the reduction in time needed to attain confluency; they continued to replicate at least through 17 PDL in 1% FBS, at which time they were transferred back to MEM + 10% FBS.

**Agglutination properties of UV-treated cells**

After at least 10 PDL in 3X growth medium, control and UV-treated cells were tested for agglutinability by exposure to varying concentrations of wheat germ lectin. UV-treated cells were agglutinated by 78 μg/ml of lectin, whereas control cultures of cells required 2500 μg/ml, more than 30 times as much (16).

**Growth in soft agar**

U.V.-treated cell populations required 20-25 PDL in the selection medium prior to seeding in soft agar. This was in contrast to 16-20 PDL for chemical carcinogen treated cells (1). When U.V.-treated cells were serially passaged through 0.35% soft agar colony frequency was 20 colonies per 10^5 seeded cells. None of the untreated cells would grow in soft agar. Occasionally, we observed small colonies of 2-6 cells in size, but they would not produce colonies 30 cells or larger and were not viable when isolated from soft agar after a 14-day incubation period in soft agar (Table 2).

**Tumor formation**

The nodules which developed at the injection site in nude mice grew to be 0.6-1.05 cm in diameter and were well-encapsulated by mouse fibroblasts (Figure 4). Removal of the tumors followed by karyological examination (1) confirmed that they were of human origin. The centers of the nodules were necrotic with dense infiltration of neutrophils and occasionally contained areas with extensive cholesterol cleft formation. Blood
vessels were present within the nodules. The nodules were compatible with a benign proliferation of exogenous cells and could most closely be described as myxofibroma-

Of 6 mice inoculated with the same inoculum, 4 developed tumors. After 4–6 days of nodule growth, the animals were sacrificed by cervical dislocation and the nodules removed for histopathology. These experiments were repeated twice. They were fixed in 10% formalin, embedded, sectioned and stained with hematoxylin and eosin. To date, similar results have been obtained in four successive attempts while 200 un inoculated control animals have not formed tumors. To date, no tumors have been observed when normal untreated cells were injected into the nude mouse (0 out of 10 inoculated mice).
Several parameters of putative changes in human foreskin cell populations during passage from the early stages of the transformation process to the late stages (growth in nude mice) were evaluated. Altered colony morphology (1), altered saturation density (1) growth at 41°C and growth in 1% FBS-supplemented growth medium were all characteristics associated with UV-irradiated cell populations as early as PDL 3 after discontinuation of the carcinogen treatment. Altered colony morphology was observed within 3 PDL after discontinued treatment. Immediately after the completion of 5 (3.2 in length) the cells were split 1:10, and part of the treated cell population was cloned at 1000 cells/cm². We observed colonies in the flasks or wells that exhibited a loss of contact inhibition and a criss-cross, disoriented piling up of the cells. These populations, when isolated from the rest of the colonies in the culture, gave rise to cell populations that exhibited abnormal morphology and an absence of the long parallel coiling growth patterns of normal untreated cultures.

After 10 PDL, irradiated cell populations exhibited an alteration in lectin agglutination profiles and grew at a temperature of 41°C. In fact, we found that 2-3 serial subpassages could be manipulated at 41°C. Normal cells will not survive 24 h at this temperature. However, at this point, the cells still would not grow in soft agar, nor were they able to produce tumors in nude mice. Serial passage to 20 PDL was required to produce populations that would grow in soft agar.

After passage through soft agar and tumor formation in the nude mouse, UV-irradiated cell populations were able to grow to a finite PDL of 120-140. Cells with a normal phenotype phased out at PDL 40 ± 5. It is interesting to note that UV light has been shown to induce carcinogenesis in the skin of man although it has weak penetrating ability. UV light can be differentiated from X-ray by the specific nature of the induced
damage and DNA repair processes. Owing to its particular features UV light, unlike many chemical carcinogens, does not require antecedent metabolic activation or specific binding before an interaction with cellular DNA.

The slopes of the toxicity profiles (survival curves) are similar to those seen for 10T1/2 cells (17) on a per cell basis (Fig. 2). When we evaluated the survival response patterns of cells at risk 12 and/or 40 h after initiating treatment (4-8 hrs prior to the reinitiation of scheduled DNA synthesis), by growing the cells at a low cell density, we found that the toxicity slopes (Fig. 3) were very similar to the slopes measured on a per cell basis (Fig. 2). We did note the presence of abnormal colonies and it was from these colonies that the neoplastically transformed cell populations arose. If we plotted the numbers of these abnormal colonies as a function of the dose in J m^{-2} recorded as a frequency relative to the number of normal colony phenotypes, the optimum numbers of transformants occurred at a survival dose -50 (E.D.50). At an E.D.65 the frequency of abnormal colonies was zero. The frequency of abnormal colonies we observed at E.D.25 was 10^{-3.5}; at an E.D.50 it was 10^{-3.0}; and at an E.D.60 it was <10^{-5}. These values were obtained for a single experiment. Repeat experiments give the same order of response. The same observations have been made for cell populations treated with chemical carcinogens of the type whose action does not require activation (1). As in animal cell systems, serially passaging these treated human cells 24 h after exposure of the cells to UV, to give them time to replicate, enhances the formation of abnormal foci.

Therefore, in order to produce cell populations in the initial stages of a transformed phenotype the transformed cells must be allowed to replicate. If the treated cells were permitted to remain in a confluent density or non-proliferating phase of growth after treatment, no transformed cell populations were obtained. Contrary to the observation by Little (18) that survival is enhanced when mouse cells are left in
growth-inhibited conditions after X-irradiation, human cells at that point are lost from transformed phenotypic populations. However, the rate of repair processes in rodent cells is much slower than in human cells, which may explain why rodent cells may be allowed a longer repair time before they replicate and establish a transformed phenotype.

Since we can optimize the transformation events by irradiating NFS cells in S, while treatment in G₁, G₂ or M minimizes the events (Milo and DiPaolo, unpublished data), we believe that the process of UV-induced carcinogenesis in human fibroblast cells is complex, error prone, and subject to critical timing of exposure to UV at an appropriate dose of the physical carcinogen. Following a selection process, the expression stage can be controlled by allowing at least 20 additional rounds of proliferation to occur before the cells will passage through soft agar and form tumors in the mouse.

In conclusion, we agree that the rate of recovery of cloning ability of human fibroblasts after UV treatment shows a correspondence with the cells' ability to recover from the potential lethal effects of the irradiation (6). However, we have found that even though we can demonstrate UV-induced transformation at doses of E.D.50 or less, we cannot demonstrate transformation at higher doses. Therefore, we feel that cell populations contain subsets of cells that are susceptible to carcinogenesis but are lost from the total cell population by modulation of the normal phenotype upon prolonged exposure to the tissue culture environment. In addition, upon repeat of these experiments, at least 5 times we have found that: 1) none of the untreated cell populations exhibited a change in phenotype, 2) out of 5 different treated populations from 5 different tissue samples, 4 of the populations could be transformed by UV radiation.
REFERENCES


J. Little, The role of cell division in the malignant transformation of mouse cells with 3MCA, Cancer Res., 35 (1975) 1637.
Time (hr)

0-24
Synchronization (PDL-5)

24-34
Sensitization

42-5
IV-Treatment

Week

1

PDL 6-10
Lectin Agglutination
Growth at 41°C
Growth in reduced serum

5

Transitional

PDL 20-25

Growth in soft agar

10
Late

PDL 34-40

14

Growth in nude mice
(Tumor)
Legend Figure 1.

The scheme followed here is a graphic representation of the sequence originating with the induction (treatment in early S) through tumor formation. Each critical stage is represented by the PDL (population doubling) reached at each stage of the process. Transition from colony formation in soft agar prior to growth in the nude mouse was achieved following isolation and seeding of colonies in 75 cm² flasks to produce 5 × 10⁶ cells of inoculum for each mouse.
Low PDL of NFS cells were seeded at 5,000 cells cm\(^{-2}\) into 60 mm diameter, 4-
1 plastic petri dishes and synchronized by amino acid deprivation. Following UV
irradiation at a fluence rate of 1.2 J m\(^{-2}\text{-sec}\(^{-1}\), the plates were incubated at 37\(^{\circ}\)C in a
1 \% CO\(_2\) humidified air atmosphere. At this time cell suspensions were prepared by
trypsinization and counted by trypan blue dye exclusion in a hemocytometer. The
closed circles represent the irradiated cultures not exposed to insulin and the closed
squares represent the irradiated cultures treated with 0.5 U/ml of insulin. Data points
are the mean values for an n of 3.
Cells were seeded at 5,000-cm$^{-2}$ into a 60 mm diameter 4-well plastic petri dish containing Dulbecco's Modified Eagle's Medium supplemented with dialyzed FBS, minus arginine and glutamine, at pH 7.2. These cells were released from the block (see text) and treated during the S phase with UV. Once the treated cells had completed S (8.2 h) they were seeded at a concentration of 200 cells-25 cm$^{-2}$ well and cloned (3) for 11 days. The control (untreated) cultures exhibited 18-24% cloning efficiencies; results are reported as a percent of control values. The circles (o - o) represent cells treated with UV. The squares (o - u) represent cells treated with UV and 0.5 U of insulin. The open circles and squares represent time points 12 h post-treatment at the conclusion of the S-G$_2$-M part of the cell cycle, while the closed circles and squares represent time points coincident with the time points used for the trypan blue experiments (Fig. 1). Each point represents data from 10 different wells.
Phottomicrograph (120X) of a serial section of a tumor excised from the subscapular region of a nude mouse. The tumor was excised 4 weeks after injection of $5 \times 10^6$ cells. The tumor was fixed in buffered formalin and stained with hematoxylin and eosin.
NFS cells (PDL 14) and UV-treated (PDL 17) passages were transferred into MEM supplemented with 1% FBS and cellular proliferation was monitored. The NFS cultures ceased dividing after 10 PDL; however, the cells from UV-treated culture appeared to adapt to the low serum concentration.
Table 1. Growth of Control (NFS) and UV-Transformed Human Cells at Reduced Serum Concentration.

<table>
<thead>
<tr>
<th>SPLIT RATIO</th>
<th>DAYS TO CONFLUENCY</th>
<th>PDL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NFS</td>
<td>UV</td>
</tr>
<tr>
<td>1:10</td>
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<tr>
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<td>3</td>
</tr>
<tr>
<td>1:4</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>1:4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>TOTALS</td>
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<td>16.6</td>
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</table>
The data presented here is an average value for colony formation for 8 wells. Each well was seeded with 50,000 cells at PDL 20 into 0.33% agar over a 2.0% agar base. The colonies were counted after 21 days and the frequency of colony growth normalized to 100,000 cells per well.
Table 2. Frequency of Colony Growth in 0.33% Agar Overlay Over a 2.0% Agar Base.

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>PDL</th>
<th>Growth in Soft Agar</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
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<tr>
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</tr>
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PRESENSITIZATION OF HUMAN CELLS WITH
EXTRINSIC SIGNALS TO INDUCED CHEMICAL CARCINOGENESIS

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Running Title: Modulation of Early Stages of Carcinogenesis
Footnote:
Abbreviations

Population doubling, PDL; fetal bovine serum, FBS; complete medium, CM; hydrocortisone, HC; 17-β-estradiol, E₂; progesterone, P; cortisone, C; Dulbecco's modified minimum essential medium, DM; Minimum essential medium, MEM; Specific activity, S.A.; 9-octadecenoic acid (oleic acid), C₁₈:₁; arachidonic acid (eicosatetraenoic acid-5,8,11,1₄), C₂₀:₄; dibutyryl cyclic GMP, d-cGMP; insulin, IN; phorbolmyristate acetate, PMA; anthralin, Anth; N-methyl-N'-nitrosoguanidine, MNNG; human nasopharyngeal carcinoma cell line, NPC; SV-40 transformed WI-26, VA-₄; Eagles Basal Medium, EBME; Low Calcium - Eagles Basal Medium medium, LoCal-EBME.
Summary

Foreskin derived low passage human cell populations were reproducibly transformed with chemical carcinogens when the cells were blocked in G\textsubscript{1}, released from the block, and treated with either the carcinogen N-methyl-N-nitrosoguanidine (MNNG) or with Aflatoxin B1 in the "S" period of the cell cycle. Arginine and glutamine deficient medium was required to effectively block the cells in the G\textsubscript{1} period. Estradiol, insulin, anthrarin or phorbol myristate acetate sensitized the cell population to carcinogen treatment when added 10 hr prior to the addition of the carcinogen in early "S" period. Presensitized cells kept blocked in G\textsubscript{1} period for 48 hr or longer, released and treated in "S" period with MNNG or Aflatoxin B1 were not transformed; nor did transformation occur in presensitized cell populations treated in G\textsubscript{2} (4.5 hrs), M (1.5 hrs) or G\textsubscript{1} (3.2 hrs). Cells derived from carcinogen-treated presensitized cells grew as colonies in soft agar at 16-20 PDL. When cells derived from colonies isolated from the soft agar were injected subcutaneously into nude mice tumors developed.
INTRODUCTION

Serially subpassaged human cells grow in vitro as randomly proliferating monolayer cultures with subpopulations capable of different rates of scheduled (Cristofalo and Sharf, 1973) and/or unscheduled DNA repair synthesis (Hart and Setlow, 1976). With continued subpassaging the non-proliferating populations constitute a progressively larger proportion of the total cell population. These changes in cell cycle kinetics with serial subpassaging could be due to a decrease in proliferating subpopulations (Cristofalo and Sharf, 1973; Merz and Ross, 1973; Turk and Milo, 1974; Milo and Hart, 1976) or to a lengthening of the G_1 or G_0 cell cycle phase (Grove and Cristofalo, 1977). In the terminal passages of Phase III unscheduled DNA repair synthesis also decreases in subcultured human diploid populations (Milo and Hart, 1976).

The expression of the transformed phenotype after a carcinogenic insult requires preferential cell multiplication. The failure of cell proliferation and fixation of the initial transformation event results in the chemically treated cells becoming part of the cell population belonging to a permanent resting phase of the cell cycle. In this way prevention or suppression of cell transformation would occur.

Previously we demonstrated that chemical carcinogen-induced transformation of human cells occurs in low passage populations that are first blocked in G_1, released from the block, and treated in "S" (Milo and DiPaolo, 1978). The current study shows that several chemicals may sensitize cells and alter the subsequent transformation response of human cells to chemical carcinogens.
MATERIALS AND METHODS

Cell Cultures

Neonatal human foreskin cell suspensions obtained by collagenase dispersion (Riegner et al., 1976), were seeded into 25-72 cm² flasks and produced confluent monolayers within 48 hrs. These cell cultures have a finite replicative capability of 35 ± 7 population doubling (PDL). Cultures containing rapidly proliferating cells (Cristofalo and Sharf, 1973) were arbitrarily assigned level 2 after the first subpassage. All populations were routinely passaged on Eagles' Minimum Essential Medium supplemented with non-essential amino-acids, sodium pyruvate, gentocin, glutamine, 25 mM Hepes at pH 7.2 (Milo and DiPaloia, 1978) and 10% fetal bovine serum (FBS). All FBS used in the above complete medium (CM) for these experiments was first analyzed for content of hydrocortisone (HC), 17-β-estradiol (E₂), progesterone (P), cortisone (C) (Milo et al., 1976), and unsaturated fatty acids (Huttner et al., 1978). FBS selected for the carcinogenesis-synchronization experiments optimally exhibited growth potential of 40-50% colony forming efficiency in cultures seeded at 250 cells/25 cm² flask.

G₁ Period

To block the human cells in the G₁ period several variations of Dulbecco's Modified Minimum Essential Medium (DM) deficient in specific amino-acids were used: lacking either leucine-glutamine, isoleucine-glutamine, leucine-arginine, isoleucine-arginine, or glutamine-arginine combinations, or one of the individual amino acids. The technique used to block the cells in the "G₁" period was modified from the Tobey and Ley procedure (1971). Cell suspensions of 5000 cells-cm⁻² were seeded onto 4 microscope slides and placed in 176 cm² petri dishes containing 50 ml of each amino acid deficient DM medium supplemented with dialyzed 10% FBS (d-FBS). Cells were
incubated in a 4% CO$_2$-enriched air atmosphere at 37°C. The cultures were released from the "G$_1$" period block by exchanging the amino acid deficient medium to growth medium (CM) consisting of Eagles' MEM 10% FBS, with the addition of $^3$H-thymidine (1 µCi/5ml) and, where appropriate to the experiment, an extrinsic factor such as IN. The radiolabeling period for continuously labeled cells was 96-144 hr (S.A. 6.0 Ci/m mole of $^3$H-thymidine) or for pulse labeled cells, 30 minutes (S.A. 60 Ci/m mole of $^3$H-thymidine). The CM, including radiolabeled thymidine, was replaced every 24 hr in the continuously labeled culture. After the block was removed, chemicals known to alter cellular DNA synthesis were suspended in CM and added to the cell population at either 24, 48, 72, 96 or 120 hr.

**Chemicals**

The extrinsic factors studied for their effect on the "S" phase of the cell cycle were oleic acid (9-octadecenoic acid (C18:1); arachidonic acid (eicosatetraenoic acid -5, 8, 11, 14 (C20:4) (Nu-Chek, Elyrian, Minn.; Huttner et al., 1978) at 5 µg/ml; dibutyryl cyclic GMP (d-cGMP) at 2.07 µg/ml; insulin (IN) at 0.5 U/ml; 17-β-estradiol (E$_2$) at 1 µg/ml; hydrocortisone (HC) (Schwartz/Mann, Orangeburg, NY) at 1 µg/ml; phorbol-myristate acetate (PMA) (Consolidated Midland Corp., Brewster, NY) at 1 µg/ml; and anthralin (Anth) (Pfaltz and Bauer, Inc., Stamford, Conn.). Chemicals were dissolved in acetone (Spectrar Grade, Mallinckrodt, Inc.) under red light in an argon atmosphere, and maintained in stock solutions of 1 mg/ml. The carcinogens, aflatoxin B$_1$ and N-methyl-N'-nitrosoguanidine (MNNG), were dissolved in acetone and stored at -19°C until immediately before use. Final culture concentration of acetone was 0.02%. Cell cultures that had been pulsed with $^3$H-thymidine (60 Ci/m mole) for 30 minutes, or continuously (6.0 Ci/m mole) for 120 hr, were acid washed with 0.1 N HCl to remove free $^3$H-thymidine. The culture slides were fixed in Carnoys' solution or
methanol: acetic acid (3:1, v/v), dipped in Kodak NTB-2 emulsion, exposed for 3–4 days at 12°C, developed in Kodak D-19 developer, fixed in Kodak fixer 197-1746, and stained with filtered Giemsa.

**Preparation of Metaphase Chromosomes**

Rapidly proliferating cell cultures at PDL 1-5 were prelabeled with $1 \mu$Ci/ml $^3$H-thymidine (6.0 Ci/m mole) for 24 hr. Grains were found on 100% of the interphase nuclei. Companion cultures radiolabeled in the same manner were fed every 24 hr with amino acid deficient DM (pH 7.2) supplemented with 10% d-FBS. Sixteen to 24 hr later the deficient medium was replaced with CM; 0.1 µg/ml colcemide was added three hr prior to fixation. Samples were removed every 30 minutes beginning four hr into the S phase, fixed in Carnoy's solution and either stained with aceto-orcein before dipped in Kodak NTB-2 emulsion or post-stained with Giemsa following development (Baserga, 1967) four days later in Kodak D-19 developer and fixation in Kodak fixer 197-1746.

**Growth in Soft Agar**

Two transformed cell lines were used as positive controls for evaluating the growth potential of chemical carcinogen-treated cells in soft agar. These were a human nasopharyngeal carcinoma cell line (NPC; gift from Litton Bionetics, Kensington, MD) and a SV-40 transformed lung cell line (VA-4; from the A.T.C.C., Rockville, MD). Both grew in soft agar (0.35% containing EBME-LoCal supplemented with 20% FBS) over a 1% or 2% agar base containing RPMI 1629 growth medium supplemented with 20% FBS (Milo and DiPaolo, 1978). The frequency of bolus formation with NPC and VA-4 after 9–11 days was 70–80% and 80–85%, respectively. The chemically treated cells were seeded at 50,000 cells/25 cm$^2$ well (Milo and DiPaolo, 1978) and examined 11 days later.
Growth in Nude Mice

Nude mice were purchased from Sprague-Dawley (Madison, Wisc.) and delivered at 6 weeks of age. Mice between 10-12 weeks of age, previously subjected to 450 R whole body irradiation, were inoculated subcutaneously with $10^6 - 10^7.5$ NPC cells-inoculum$^{-1}$ into the subscapular region. After 24 hrs the initial bleb regressed and 4-6 weeks later a tumor 0.6-1.2 cm in size was excised. The optimum inoculum size was $5 \times 10^6$ cells. VA-4 cell inocula regressed in 24 hr and no visible tumor was seen in the 20 inoculated mice after 6 month period. The tumor incidence in NPC-inoculated mice was 3/10. The chemical carcinogen-treated cell populations were inoculated at a cell inoculum size of $5 \times 10^6$ cells in 0.25 ml volume in the manner previously described and submitted for pathology (Milo and DiPaolo, 1978). After 4-6 weeks the tumors were removed and submitted for pathology.

RESULTS

Cell Block

Cells from amino acid-deficient DM preparations supplemented with 10% d-FBS contained varying numbers of radiolabeled nuclei (Fig 1). At 96 hr cells maintained in minus glutamine and arginine (Medium A), minus leucine and glutamine (Medium B) or minus isoleucine and glutamine (Medium C) contained 5, 37 and 57% radiolabeled interphase cells, respectively.

In DM deficient media lacking only one amino acid (i.e., arginine, glutamine, or isoleucine) the cell populations contained 50-70% radiolabeled interphases in a 96 hr period. A 65% increase in cell numbers 10 hrs after the S period responded to the number of radiolabeled interphase nuclei observed.
Medium A was selected for blocking the cells in G1, and samples were checked at two-hr intervals. Cell populations seeded for 24 hr in medium A and transferred to CM for 72 hr contained 67-72% radiolabeled nuclei. Cells kept in medium A for 48 hr prior to transfer to CM contained 35-42% radiolabeled nuclei at the end of an additional 72 hr period. The area in Fig. 2 between the curves for randomly proliferating cell populations (open circles) and the cells held in medium A for 24 hr (filled circles) represents cells that respond to IN, Anth, E2, or PMA treatment (see below). The area described by the cell population that was held in medium A for 48 hr (Fig. 2, triangles) represents a population that will not totally respond to added signals such as IN, Anth, or E2.

**Effect of Exogenous Factors on Cell Growth**

Cell populations were transferred from medium A to CM after 24 hr and one of the following chemicals was added: IN, HC, C20:4, E2, d-cGMP, PMA, or Anth (Fig. 3), along with 3H-thymidine. In these experiments, untreated control cultures transferred to CM after 24 hr in medium A contained radiolabel in only 62-67% of cell nuclei up to 120 hr after transfer. Either IN, C20:4, E2, d-cGMP or HC added to CM amplified the appearance of radiolabeled nuclei over the 120 hr sampling period. Anth or PMA did not alter the profile of 3H-thymidine incorporation into nuclei. IN-treated cells recovered from the block more rapidly than any of the other cultures. Whenever treated cultures were removed from the experimental medium and passaged, the normal proliferative kinetics resumed by passage 2 or 4. The PDL time after a 1:2 split was approximately 2-3 days at PDL 2. In no case was the lifespan of cultures appreciably altered from 37 ± 7 PDL.

To further study the effect of these compounds on the proliferative capability of the cells, populations were maintained in medium A for 48 hr (Fig. 4) before
treatment. Untreated control cells in these experiments contained 43% radiolabeled nuclei 48 hr after transfer from medium A. IN-treated cell populations contained 72% radiolabeled nuclei. C20:4- or HC-treated cultures sampled for up to 120 hr incorporated $^3$H-thymidine into no more than the 72% of nuclei labeled at 48 hrs. Again, in Anty- or PMA-treated cultures the labeling profile did not differ from the untreated cultures.

Since it was difficult to predict the exact length of the lag interval before the cells in $G_1$ would begin to enter the "S" period following transfer from medium A to CM, the cells were blocked in $G_1$ in medium A for 24 hr and then released. At point B (Fig. 5) either IN or one of the other compounds (See Figs. 1-3) were added to the CM. At 30 minute intervals for the next 25 hr, sample populations on 1 mm were removed and placed in CM containing 1.0 µCi of $^3$H-thymidine (60 Ci/m mole) in 5 ml for 30 minutes. Untreated control cultures achieved a level of only 73% radiolabeled nuclei, whereas cells treated with IN reached a 90-95% level.

When E$_2$, Anth or d-cGMP (Table 1) were added to pulse labeled cultures, the "S" peak occurred 1 hr later. Compared to untreated controls, the length of S (i.e., the appearance and disappearance of labeled interphase nuclei) did not vary in any of the treated populations; the length of "S" in all populations was 8.2 hr. The length of M, 1.3 hr, was determined by measuring the interval for the appearance and disappearance of radiolabeled metaphase nuclei. The interval between "S" and M ($G_2$) was calculated to be 4.5 hr. The length of the cell cycle, 22.4 hr, was determined by counting cells over a 24 hr period using trypan blue dye exclusion. Subtraction of the experimentally determined "S" and M times and the calculated time for $G_2$ from 22.4 yielded an estimated $G_1$ of 8.2 hr. If either IN or Anth was added at interval B (Fig. 5) the period most dramatically affected was $G_1$. $G$ was shortened from 8.2 to 6.5 hr between
waves of cells passing through "S", G₂ and M for three cycles. After the first cycle and for each successive cycle 20-22% of the cells departed from the synchrony pattern. After the second cycle the number of rapidly pulse-labeled nuclei decayed to that of randomly proliferating companion cultures. The absolute values for the total number of cells in "S" varied with tissue and according to treatment. When experimental sister cultures were treated with PMA, IN, Anth or E₂ at point B (Fig. 5) the number of cells in "S" varied from 67-95% (Table 1).

**Cell Transformation Studies**

Ten hr after administration of IN or a compound listed in Table 1 at interval B (as the cells were entering "S"; Fig. 5), aflatoxin B₁ or MNNG was added to the cultures. The carcinogens were removed 12 hr later and the cultures serially passaged into CM containing 8X non-essential amino acids and 2X vitamins (Milo and DiPaolo, 1978), for 15 PDL. The populations were then serially passaged into soft agar (0.35%, containing EBME-LoCal, supplemented with 20% FBS) over a 2% agar base containing RPMI 1629 growth medium supplemented with 20% FBS (Milo and DiPaolo, 1978). After 11 days colonies were scored, removed and serially subpassaged in CM. Growth in soft agar for treated cultures ranged from no growth to $1:10^{2.5}$ for aflatoxin B₁-anthralin treated cultures (Table 2). Cell populations treated with non-carcinogenic compounds and passaged through soft agar sometimes formed 2-6 cell short chains rather than a colony. None of these cells formed colonies in agar when passaged a second time. Addition of IN, E₂, PMA or Anth at interval B enhanced colony formation in soft agar as compared with aflatoxin treatment alone. Serially passaging the cell a second time in the agar increased the frequency of colony formation to 40-90%. Similar results recurred with MNNG-treated cell populations regardless of whether IN or Anth were used (data not shown here). PMA-aflatoxin B₁ treated
populations, while exhibiting a low frequency of colony formation upon passage through the soft agar for the first time, did form colonies of 50-300 cells per colony, followed by an increase in frequency in colony formation to 70% during the second agar passage.

Addition of oleic acid (C18:1), C20:4, or d-cGMP did not enhance colony formation. E₂ and PMA were intermediate in their effect on colony formation by carcinogen-treated cells, while IN or Anth were quite effective in augmenting colony formation (Table 2). After carcinogen-treated cells isolated from agar were serially passaged, 5 x 10⁶ cells were inoculated subcutaneously into the subscapular region of previously irradiated (450R whole body) nude mice (Milo and DiPaolo, 1978). Subcutaneous tumors appeared at the injection site within 10-18 days. The tumors were scored 4 weeks later when 0.8-1.5 cm in size. Cell populations treated with aflatoxin B₁-IN or Anth, or with MNNG-IN or Anth, produced the highest numbers of tumors (Table 3). In three experiments (data shown for one experiment in Table 3) the order of successes was the same. Aflatoxin B₁-IN or MNNG-IN induction of tumors was the highest, followed by Aflatoxin B₁-Anth, or -PMA. Tumors excised and examined histopathologically and karyologically were all confirmed as undifferentiated mesenchynal tumors of human origin. To date, all cell lines that have been grown in soft agar, and successfully serially passaged a second time in soft agar, have produced tumors when injected in nude mice. No cell populations after being blocked for 48 or 72 hr and treated in "S" with Aflatoxin B₁-IN or MNNG-IN formed colonies in soft agar or produce tumors in nude mice.

DISCUSSION

As previously reported (Milo and DiPaolo, 1978), we have successfully induced transformation of normal human cells with different chemical carcinogens. Other
reports suggest that randomly proliferating normal skin cell populations can be transformed by 4-nitroquinoline oxide and MNNG. However, we have found that successful induction of neoplastic transformation in rapidly proliferating normal human foreskin cells by chemical carcinogens in vitro is an exceedingly rare event. Chemicals that act as carcinogens in neonatal foreskin cells in vitro can damage cellular DNA (Milo et al., 1978), but the DNA repair systems in these normal diploid cell populations are extremely rapid and error-free (Maher et al., 1977).

Randomly proliferating normal cell populations repair over 90% of the damage from chemical carcinogens in 4-10 hr (Milo and Hart, 1976) and are rarely transformed. There are two methods for synchronizing mammalian cells in G1 phase of the cell cycle; one is to arrest the cells in confluence and the other is amino acid deprivation (Peterson et al., 1974; Jones et al., 1973; Jones et al., 1977; Grisham et al., 1979). Following release from the block nearly all the cells enter "S" phase within 6 to 8 hr. C3H 10T1/2 cells arrested in this manner, released and treated with MNNG in "S" phase exhibit an increase in sensitivity to MNNG and become more readily transformed. However, isoleucine deficient medium does not block human cells in G1. Furthermore, release of human cells from block by subpassaging the cells, after density dependent inhibition in G1 with carcinogens over a 10 hr period, does not result in a carcinogen-induced transformation event. In fact, the transformation of human cells at early passage levels is inhibited if the transformable cells are kept in a density-inhibited state for 8-16 hr prior to subpassaging. Moreover, cultures held in the G1 phase of the cell cycle for more than 24 hr cannot be transformed when treated with aflatoxin B1 or MNNG in the "S" phase. A double amino acid deficient medium (minus glutamine and arginine) was necessary to adequately block the cells in G1. Augmentation of the transformation occurs when IN, Anth, E2, or PMA are added to
the cultures prior to carcinogen administration. The major difference between the effectiveness of aflatoxin B and MNNG as carcinogens appears to be the time at which the compounds are added to the cells entering "S". MNNG is more effective if added early in "S" (0-4 hr after recovery from the amino acid block) while aflatoxin B, is more effective if added from 2 hr prior to "S" to 4 hr into "S". The incidence of transformation was reduced to less than 1 out of 10 when these compounds were added to the cells in late "S" (4 to 8 hr into S).

IN, PMA, E, and Anth have been found to modulate the expression of other transforming agents, and the effects on chemical carcinogen-induced carcinogenesis are not unique. Enhancement of virus-induced transformation by some of the same chemical factors used here occur with E (Milo et al., 1972), HC (Schaller et al., 1976), and PMA (Weinstein et al., 1979), these chemicals can interfere with semi-conservative DNA synthesis (Milo and Hart, 1975). Modulation of cellular functions is not an unusual response by cells to the presence of these compounds and they obviously facilitate transformation by chemical carcinogens. This suggests that, as in the 3T3 system described by Grisham et al. (1979), that human cell subpopulations are sensitized in "S" to carcinogens by amino acid deprivation and reconstitution. This sensitization is enhanced by Anth, IN, PMA or E at pharmacological concentrations of the drugs.

Many of the compounds studied here (E, HC, C20:3, C20:4, IN) are components of fetal bovine serum and serve as natural regulatory agents of proliferation, the action of PMA has been suggested to resemble that of hormonal agents. The pleiotypic responses of subpopulations to the carcinogen treatment may be induced by pretreatment with PMA, E, Anth or IN. Therefore, after subsets of populations have
been modulated by being kept in $G_1$ for 48 hr or serially subpassaged in vitro to PDL >5, the cells no longer respond to the extrinsic factors in the presence of the carcinogens.
ACKNOWLEDGEMENTS

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We wish to thank Mr. Robert Zimmerman, Mr. Thomas McMichael and Mrs. Inge Noyes for their expert technical assistance in cell preparation and their participation in the cell cycle studies.
TABLE 1

PERCENT $^3$H-THYMIDINE LABELED NUCLEI DURING THE PEAK TIME INTERVAL OF SCHEDULED DNA SYNTHESIS OF CELL POPULATION TREATMENT WITH DIFFERENT EXTRINSIC FACTORS.

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>C20:4</th>
<th>IN</th>
<th>HC</th>
<th>$E_2$</th>
<th>Anth</th>
<th>PMA</th>
<th>d-cGMP</th>
<th>Untreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>34</td>
<td>35</td>
<td>42</td>
<td>45</td>
<td>21</td>
<td>22</td>
<td>17</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>35</td>
<td>74</td>
<td>77</td>
<td>74</td>
<td>33</td>
<td>33</td>
<td>23</td>
<td>47</td>
<td>49</td>
</tr>
<tr>
<td>36</td>
<td>90</td>
<td>92</td>
<td>89</td>
<td>51</td>
<td>41</td>
<td>35</td>
<td>67</td>
<td>72</td>
</tr>
<tr>
<td>37</td>
<td>84</td>
<td>81</td>
<td>81</td>
<td>91</td>
<td>33</td>
<td>72</td>
<td>67</td>
<td>67</td>
</tr>
<tr>
<td>38</td>
<td>63</td>
<td>72</td>
<td>72</td>
<td>67</td>
<td>38</td>
<td>15</td>
<td>63</td>
<td>54</td>
</tr>
<tr>
<td>39</td>
<td>57</td>
<td>60</td>
<td>57</td>
<td>37</td>
<td>22</td>
<td>7</td>
<td>55</td>
<td>34</td>
</tr>
</tbody>
</table>

Concentrations used were below level that inhibited cellular proliferation as evaluated by relative plating efficiency of the cell population (Milo et al., 1976).
Legend for Table 2

The carcinogenic activity of Aflatoxin B1 in cultures in the presence of added factors that altered the response pattern are presented. The exogenous factors selected for this study were insulin, (IN); estradiol, (E2); arachidonic acid, (C20:4); oleic acid (18:1); defutryl cyclic GMP, (d-cGMP); phorbol myristate acetate, (MPA); and anthralin, (Anth). Each of these factors was added at point B as described in Fig. 5. Ten hr later as the cells were entering "S" aflatoxin B1 was added to the cells. After the treated cells had passed through "S" both the cultures treated with the factor alone and/or carcinogen and factor were removed (18.3 hr) after point B (Fig 1). Column 1 presents the concentration of the carcinogen and factor added to the cells. Column 2 presents the number of lines that grew in soft agar/number of lines seeded in soft agar. Column 3 presents the frequency obtained per number lines that grew in soft agar. Frequency is the number of colonies formed in 21 days per 50,000 cells seeded into at 0.35% agar overlay (Milo and DiPaolo, 1978). The results were expressed as log values to the base 10.
TABLE 2

FREQUENCY OF COLONY GROWTH IN SOFT AGAR OF TRANSFORMED HUMAN CELLS TREATED WITH DIFFERENT EXTRINSIC FACTORS AND AFLATOXIN B₁.

<table>
<thead>
<tr>
<th>Chemical (µg/ml)</th>
<th>No. of Treated Populations</th>
<th>No. of lines that grew in S.A./No. of lines seeded in S.A.</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Aflatoxin-B₁ (10)</td>
<td>10</td>
<td>3/20</td>
<td>$1 \times 10^{5.2}$</td>
</tr>
<tr>
<td>Aflatoxin-B₁ (10) - IN (1)</td>
<td>10</td>
<td>10/10</td>
<td>$1 \times 10^{5.1}$</td>
</tr>
<tr>
<td>Aflatoxin-B₁ (10) - E₂ (1)</td>
<td>2</td>
<td>2/2</td>
<td>$1 \times 10^{6.0}$</td>
</tr>
<tr>
<td>Aflatoxin-B₁ (10) - C20:4 (1)</td>
<td>3</td>
<td>3/3</td>
<td>$1 \times 10^{5.2}$</td>
</tr>
<tr>
<td>Aflatoxin-B₁ (10) - C18:1 (1)</td>
<td>3</td>
<td>1/3</td>
<td>$1 \times 10^{5.6}$</td>
</tr>
<tr>
<td>Aflatoxin-B₁ (10) - d-cGMP (1)</td>
<td>2</td>
<td>1/2</td>
<td>$1 \times 10^{5.7}$</td>
</tr>
<tr>
<td>Aflatoxin-B₁ (10) - PMA (1)</td>
<td>5</td>
<td>5/5</td>
<td>$1 \times 10^{6.7}$</td>
</tr>
<tr>
<td>Aflatoxin-Anth (1)</td>
<td>4</td>
<td>4/4</td>
<td>$1 \times 10^{2.3}$</td>
</tr>
<tr>
<td>Anth (1)</td>
<td>3</td>
<td>1/3</td>
<td>$1 \times 10^{5.2}$</td>
</tr>
</tbody>
</table>
TABLE 3

EFFECT OF EXTRINSIC FACTORS ON GROWTH OF TRANSFORMED CELLS IN SOFT AGAR AND TUMOR FORMATION IN THE NUDE MOUSE.

<table>
<thead>
<tr>
<th>Treated Cell Populations</th>
<th>Extrinsic Factors</th>
<th>*Frequency of Growth in Soft Agar</th>
<th>*Growth in Nude Mouse</th>
<th>**No. of successes/No. of attempts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxin B₁</td>
<td>none</td>
<td>+</td>
<td>+</td>
<td>1/3</td>
</tr>
<tr>
<td></td>
<td>IN</td>
<td>+</td>
<td>+</td>
<td>7/7</td>
</tr>
<tr>
<td></td>
<td>Anth</td>
<td>+</td>
<td>+</td>
<td>5/7</td>
</tr>
<tr>
<td></td>
<td>PMA</td>
<td>+</td>
<td>+</td>
<td>2/8</td>
</tr>
<tr>
<td></td>
<td>E₂</td>
<td>+</td>
<td>+</td>
<td>4/11</td>
</tr>
<tr>
<td></td>
<td>C₂₀:₄</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C₁₈:₁</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>HC</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>d-cGMP</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>none</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

| MNNG                     | none             | +                                | +                     | 1/11                             |
|                          | IN               | +                                | +                     | 4/5                              |
|                          | Anth             | +                                | +                     | 5/5                              |
|                          | PMA              | +                                | +                     | 3/9                              |
|                          | E₂               | +                                | +                     | 2/8                              |
|                          | C₂₀:₄            | -                                | -                     |                                  |
|                          | C₁₈:₁            | -                                | -                     |                                  |
|                          | HC               | -                                | -                     |                                  |
|                          | d-cGMP           | -                                | -                     |                                  |
| None                     | none             | -                                | -                     |                                  |

*The dashes (—) indicate there were no observable responses (bolus formation of less than 50 cells) within 14-21 days in soft agar and no response within 6 months for growth of the cells isolated from soft agar. Transformed cells were injected at a cell concentration of 5 X 10⁷ cells/0.5 ml into the subscapular region of the nude mouse. The tumors were allowed to grow to 0.6-1.2 cm in size (Milo and DiPaolo, 1978). Soft agar is defined as a 0.35% upper layer of agar containing growth medium on a 1% or 2% agar base (see text for details). Control (untreated cell) inocula did not grow in soft agar and when injected directly from culture (10⁷ cell-inoculum)⁻¹ into the mouse the bleb(s) regressed in 24 hr.
Human foreskin cell populations were seeded at a cell density of 3,000 cells cm$^{-2}$ onto a 15 mm diameter microscope slide or a coverslip. These seeded slides were then placed into 25 cm$^2$ dishes containing 5 ml of DM supplemented with 10% d-FBS minus arginine-glutamine (●●), minus leucine-glutamine, (▲▲), or minus isoleucine-glutamine (○○), and monitored for the presence of radiolabeled interphases for 96 hr after seeding. The deficient medium was replaced every 24 hr. $^3$H-thymidine (1 Ci/5ml; S.A. 6 Ci/m mole) was added at the time the cells were seeded into the DM. The radiolabel was replaced every time the DM or CM was replaced. Four samples were taken at 1 hr over the 96 hr period, fixed and prepared for autoradiography (see text).
Figure 1

% Labeled Interphases

TIME (hr.)
Human foreskin cell populations at saturation density were seeded at a cell density of 5000 cells-cm$^{-2}$. These populations were not permitted to remain in saturation density for more than 16 hr prior to seeding. The cells were suspended by trypsinization (Riegner et al., 1976) and the cells recovered by centrifugation at 650 X g. The pellet was resuspended into experimental medium A and seeded onto 13 mm diameter microscope sides or coverslips for 6 hr to attach to the substratum. These cultures were kept in this deficient medium for 24 hr (o-o) (see Legend 1) or 48 hr (Δ-Δ). The block was removed by the addition of 10% FBS-supplemented CM containing 2 mM arginine and 1 mM glutamine. One micro curie of $^3$H-thymidine (6.0 Ci/m mole)/5 ml was added to each amino acid deficient experimental medium and each complete growth medium. In each case both medium and radiolabel thymidine were replaced at each and every 24 hr period from seeding to completion of the experiment. Cells kept continuously in CM and serially subpassaged into CM were sampled every 2 hr from 0 hr up to 96 hr (o-o) post-seeding.

Proliferating cell populations seeded into experimental medium A for 24 hr (o-o), 4 samples were removed every 2 hr from 0 hr (at 24 hr time pt) up to 96 hr. The block was removed at 24 hr. Additional populations were left in experimental medium up to 48 hr (Δ-Δ) the block removed. Another population was left in the amino acid-deficient medium from 0 hr up to 120 hr (Δ-Δ). The medium plus radiolabel thymidine was replaced every 24 hr.
Fig 2

% LABELED INTERPHASES

TIME (hr.)

0 10 20 30 40 50 60 70 80 90 100

0 8 16 24 32 40 48 56 64 72 80 88 96 104 112 120
Legend - Figure 3

Randomly proliferating cell populations that were not in saturation density arrest for more than 16 hr were seeded into CM (o-o) or medium A (■-■) and allowed to attach for 6 hr (≥95% attachment was determined by fixation, staining and counting the number of cells that attached to the substratum versus the number of cells seeded). All populations seeded into medium A were transferred to CM plus 2 mM arginine and 1 mM glutamine and either HC, C20:4, E2 (▲-▲); or IN (▲-▲); or PMA (e-e); or Anth (e-e); or d-cGMP (□-□) added to each separate culture at 24 hr. Radiolabel thymidine was added to the cultures as described in the legend for Fig. 2. Cultures were refed every 24 hr over the 120 hr period.
Legend - Figure 4

Cell populations used in these experiments were prepared and seeded in the same manner as described in the legend for Fig. 3. However, these cultures were released from block after 48 hr. Radiolabel thymidine was added to medium A and/or CM as described in the legend for Fig. 2. Control cultures (o-o) were released after 48 hr and IN (o-o) or HC (Δ-Δ) added. Other compounds listed in the legend of Fig. 3 induced responses intermediate between IN (o-o) or HC (Δ-Δ) treated cultures. These compounds were added to CM at 48 hr after replacement of medium A. The untreated control cultures were left in medium A (Δ-Δ). Radiolabel thymidine was added to the cultures at seeding and every 24 hr upon replacement of medium A or CM.
Legend - Figure 5

Cell populations were seeded at a cell density of 5,000 cells-cm^{-2} with DM minus arginine-glutamine, (A) supplemented with 10% d-FBS. The cells were fixed 24 hr later, stained with hematoxylin, and enumerated (continuously 95% absolute plating efficiency occurs). The DM was removed at this time and the cultures released with CM (see text) containing 0.5 U of insulin per ml. Companion wells containing 5 ml of CM with $^{3}$H-thymidine were incubated under identical culture conditions as for the experimental DM cultures. One microcurie of $^{3}$H-thymidine (60 Ci/m mole^{-1}) was added. At 30 minute intervals samples were removed from DM or CM medium, incubated for 30 minutes in the radiolabeled CM medium, fixed, stained and developed under NTB-2 emulsion for 4 days. The labeled interphases were enumerated. Companion slides and labeling conditions were used in controls except 0.1 ug/ml of colemide was added to the CM medium. Four hrs into "S", samples were taken and radiolabeled for 30 minutes in CM, fixed, and developed under NTB-2 emulsion (see text). Radiolabeled metaphases were enumerated and the percentages compared to the controls were calculated (not reported here).
Fig. 5

% Labeled Interphases

TIME (hr.)

0 4 8 12 16 20 24 28 32 36 40 44 48
REFERENCES


JONES, P., BENEDICT, W., BAKER, S., MONDAL, S., RAPP, U., BERTRAM, J.; and BENEDICT, W., Cell cycle-specific oncogenic transformation of C3H/10T1/2 clone 8


Segment II

Work Scope - 1979

1. Original Objectives

1. Determine whether suspect fuels with carcinogenic potential inhibit or enhance ST-FeSV virus-directed transformation of human cells in a predictable manner and correlate effects on virus transformation with \textit{in vitro} chemical transformation in Segment I.

2. Investigate activation of endogenous human virus by suspect carcinogens.

3. Investigate hormonal influence on chemical-viral interactions.

4. Develop techniques and procedures to be used in hybridization analysis of chemically treated ST-FeSV virus-infected cells.

Specific Objectives of Segment II

1. Determine whether napthylamines, hydrazine (HZ), monomethylhydrazine (MMH), unsymmetrical dimethylhydrazine (UDMH) and symmetrical dimethylhydrazine (SDMH) enhance or inhibit Snyder-Theilen Feline Sarcoma Virus directed cell transformation of human skin cells \textit{in vitro} in a predictable manner.

2. Determine whether HZ, MMH, UDMH and SDMH activate endogenous C-type RNA virus expression in human cells.

3. Develop techniques, procedures and materials for hybridization analysis of HZ, MMH, UDMH, SDMH and BP treated ST-FeSV infected human cells for increased or decreased genome expression.
11. Progress to Date

1. Determined alpha-napthylamine (ANA), phenyl-alpha-napthylamine (PANA) and phenyl-beta-napthylamine (PBNA) significantly inhibited ST-FeSV transformation of human skin fibroblasts.

2. Determined HZ, H4H, UDMH and SDMH Inhibited ST-FeSV transformation. Inhibition was dependent upon temporal relationship of treatment to infection.

3. Results of co-carcinogenesis assays showed high degree of correlation with in vitro chemical transformation and neoplastic transformation in Segment 1.

4. Determined long-term exposure of human skin fibroblasts (HSF) to HZ, UDMH, M4H (FY 78) and SDMH (FY 79) did not activate human endogenous virus.

5. Determined LD50 for shale oil and petroleum derived JP5 and DFH fuels.

111. Introduction and Background

Hydrazine, hydrazine derivatives, and polycyclic hydrocarbons are of increasing biological concern because of their potential to act as mutagens or carcinogens. Thus, efforts must be concentrated toward developing rapid, sensitive and inexpensive biological assays which would determine carcinogenic and mutagenic potential of these compounds.

While many biologic effects of hydrazine fuels and its derivatives have been studied in animals, the extrapolation of these biological effects to man has been difficult because of differential responses manifested in diverse species of test animals. Prolonged exposure of test animals to these compounds has suggested potential tumorigenic and carcinogenic activity with a diverse number of tumor types noted, depending upon the species and fuel component used. For example, Diwan et al. (1) 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.

Rats, mice (2,3), syrian hamsters (4) either fed or injected with HZ and its derivatives, developed different types of tumors or leukemias depending upon the route of administration, the compound used and the species involved in the assay. Pulmonary tumors, reticulum cell sarcomas, myelogenous leukemia and histocytomas are some types of neoplastic diseases observed in these test animals.

Additionally, the cost of maintaining animals during the long latency period required for manifestation of the carcinogenic event to occur, is considerable. Thus, the evidence from animal assay systems suggest that long-term exposure of these fuel components may pose a potential carcinogenic threat to man.

In this segment of the contract, we proposed to evaluate the molecular interactions of HZ, MMH, SDMH, UDMH, ANA, PANa, PBNA and BP with oncogenic viruses in human cells and to correlate this effect with in vitro chemical transformation proposed in Segment I. We have used Snyder-Thilen Feline Sarcoma Virus (ST-FeSV), an oncogenic-RNA virus which transforms human cells in vitro in measurable and predictable dose response kinetics (5).

The importance of this study may be the development of an in vitro assay system with human cells that can quantitatively screen potential co-carcinogens within a short-period of time (9-13 days). All chemicals not having a carcinogen capability may indeed be co-carcinogens. This procedure should identify those components in jet and rocket fuel that are co-carcinogens.
We previously showed that combinations of hormones, carcinogens and an oncogenic DNA virus, SV40, enhanced virus transformation depending upon dose and time of application (6, 7). We concluded from these studies that at least certain chemical carcinogens enhanced viral transformation by increasing the number of sites for integration of viral genetic information into cellular DNA (6).

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

The above observations with a non-replicating virus system (oncogenic DNA virus) have not been as clearly substantiated with replicating oncogenic RNA virus systems. Nonetheless, Freeman and Price et al. (8, 9) have independently demonstrated that murine leukemia virus infected rat and mouse cells undergo transformation following addition of carcinogens such as 3-methylcholanthrene (MCA), BP, and diethylnitrosamine (DENA). The implications of their studies are that the carcinogens activate the viral coded oncogenic information which may be inherent in the cells but require the helper functions of the leukemia virus. However, others (8, 9) found that treatment of rat embryo cultures with MCA one to three weeks prior to addition of Rauscher leukemia virus (RLV) did not lead to transformation of these cells, whereas MCA treatment up to three weeks after RLV infection yielded transformation within 7 to 10 passages. These data indicate that the 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.
One advantage of the RNA tumor virus system is that the temporal relationship that exists between addition of virus and chemical in the oncogenic DNA virus system is greatly diminished. Because the virus continues to replicate, chemicals may be added simultaneously, shortly after or at some later time to the ST-FeSV virus infected cell.

The evidence that many carcinogens enhance oncogenic DNA viral transformation in vitro suggested similar approaches be undertaken with human cells and HZ, MMH, UDMH, SDMH, ANA, PANA and PBNA. In this laboratory, the chemicals and carcinogens demonstrated to enhance viral transformation of hamster, mouse and rat cells were examined for similar effects with SV40 virus (7) and were examined with ST-FeSV (10). Our studies to date have revealed a good correlation between the ability of an in vivo carcinogen to enhance oncogenic DNA virus transformation chemicals included in this group, include BAP, MNNG, 4NQO, Acetoxy AAF, but not MCA nor 7,12 DMBA (7). However, the results found with the ST-FeSV human cell transforming system demonstrated a marked inhibition of virus directed transformation (10-12).

Endogenous C-type RNA viruses have been observed by electron microscopy in 18 different mammalian species including humans, several avian species, snakes and fish (13). Their presence as integrated genomes in vertebrates suggested these viruses served as regulators of natural life processes (14).

Endogenous C-type virus release has been shown by Panem et al. (15), to occur sporadically in normal human fibroblasts and evidence has been provided by this group, that endogenous viruses may play a role in at least one human autoimmune disease. Further, the expression of endogenous virus genes appear to be under cellular control. We have recently shown using
FeSV transformation of human cells, that select chemical carcinogens may interfere with host cell translational mechanisms, proviral synthesis or integration (16).

Since carcinogens may interfere with these functions, they may also interfere with gene products that function as suppressors of endogenous virus expression. Thus, normal cells treated with chemical carcinogens may induce expression of endogenous viruses whose presence may be detectable by electron microscopy, simultaneous detection of 70S viral RNA and RNA directed DNA polymerase, or immunological techniques.

In murine cells, endogenous viruses induced by halogenated pyrimidines or protein inhibitors, may be detectable from 12 hours to 96 hours post treatment. Thus, if endogenous virus release is detectable after chemical treatment and correlated with carcinogenic potential of the chemical, a rapid sensitive assay may be developed to screen potential carcinogens.

Steroid hormones have been reported to inhibit viral infection \textit{in vitro} (19), enhance or inhibit viral oncogenesis in animal species (20), enhance or inhibit viral transformation \textit{in vitro} (6,22), modulate oncogenic RNA virus expression in non-permissive cells (23) and to inhibit DNA repair (excision) with increased virus transformation in estrogen and chemical carcinogen treated SV40 virus infected human cells (6).

The mechanisms of hormonal action suggest the hormone enters the cell by diffusion, where it binds with low molecular weight proteins or receptor molecules present in cell cytoplasm. The hormone-receptor complex with increased affinity for chromosomal sites is translocated to the nucleus, whereby an undetermined mechanism, gene expression, is modulated.
Since hormones modulate gene expression and have been shown to affect oncogenic expression of both chemical transformation (Milo Segment 1, this report) and virus transformation (6), their interaction with known carcinogens which may interfere with host cell translational mechanisms may result in endogenous viral expression in human cells.

IV. Rationale

Excellent reviews on the molecular biology of transformation of mammalian cells by oncogenic RNA and DNA viruses have been written and will not be discussed at length here (17-19).

Almost without exception, malignant transformation requires that at least a segment of the infecting viral genome, in the case of RNA viruses, the DNA provirus, becomes integrated into the host cell genome. Integration requires an alteration in at least the primary structure of cellular DNA. For transformation to occur, cellular DNA synthesis following viral integration is required (18).

A major difference in cellular transformation by oncogenic viruses and chemical carcinogens is that new genetic information is added to the cell by the former and existing genetic information is modified by the latter. However, the modification of genetic material by chemical carcinogens may also activate (derepress) pre-existing endogenous oncogenic viral genes.

A. Feline Sarcoma Virus. The Snyder-Theilen strain of Feline Sarcoma Virus produces progressively growing sarcomas in cats, dogs, sheep, rabbits, pigs, rats and monkeys (23-29). Cells from these species as well as from oxen (30) and humans (31-34) are transformed in vitro.
FeSV is an oncornavirus, and like other such viruses, stimulates cellular DNA synthesis during transformation of infected cells. Although it has not been shown directly that FeSV like other oncornaviruses forms a DNA pro-viral replicate that becomes integrated in the cell genome, it seems a reasonable assumption that this also occurs with FeSV (35). Indirect evidence supporting this assumption is the formation of sarcoma positive, leukemia negative (S+L-) mouse cells transformed by Moloney murine sarcoma virus (MMSV) which produces lytic foci in reaction to infection by ecotropic MuLV (36). A similar S+L- cell line has been produced by infection of cat cells with MMSV and is insensitive to both replicating MuLV (xenotropic) and feline leukemia virus (37).

Criteria commonly used for determining genetic expression in transformed cells (endogenous and exogenous) C-type RNA viruses, has been the serologic detection of specific (38) or interspecies specific antigens (39). With regard to transformation by C-type RNA viruses, the expression of tumor-specific antigen (FOCHA) (39), abnormal colonial morphology or foci of transformed cells as expressed by loss of contact inhibition and/or growth in soft agar, have been used as a means to judge the transformation event (39,40).

This laboratory has demonstrated the reproducibility of in vitro transformation (focus formation) by Snyder-Theilen Feline Sarcoma Virus (ST-FeSV) in Detroit 550 human skin fibroblasts. Transformed foci appeared as rounded hyper-refractile cells and were observed to follow the pattern of monolayer cell growth. Giant cell formation was noted 14 days p.i.

Reproducibility of this cell-virus system permitted us to study the role chemical carcinogens and oncogenic RNA viruses play in transformation.
Because of our extensive experience with this virus in vitro and in vivo, we choose to proceed with it in lieu of other RNA sarcoma viruses. This approach is predicated on the basis that the mechanisms involved in promotion of RNA tumor virus transformation by chemicals or vice versa should be nearly identical with all RNA tumor viruses.

The use of an RNA tumor virus in co-carcinogenesis studies is justified on the basis that the process of integration of viral genetic information, in order for transformation to occur, is more complex with an RNA tumor virus. The requirement for synthesis of a DNA copy or copies of the RNA genome will certainly alter time sequences between addition of carcinogen and virus as previously discussed.

B. Activation of C-Type Viruses by Chemical Carcinogens. The evidence for C-type RNA virus activation in mammalian and avian cells by various means warranted analyses for endogenous RNA C-type viral expression in human cells transformed by SV40 virus or treated with hydrazine and derivatives and polycyclic hydrocarbons. A number of investigators have reported induction of virus synthesis in normal cells by chemical, physical, and viral carcinogens. Leukemia virus group-specific antigen (HaLV gs) in hamster cell lines transformed by MCA or by certain fractions of cigarette smoke condensates has been demonstrated. The transformed cell lines were negative for infectious virus before inoculation into hamsters. However, hamster-specific C-type RNA virus was isolated from tumors or from cell lines derived from the tumors. Since C-type viruses were normally not found in either hamster tissue or hamster tumors, they concluded the chemical treatment activated the virus.

Rowe et al. (41) reported the establishment of a virus-negative AKR mouse embryo cell line which could be induced to yield virus by X-irradiation,
ultraviolet irradiation, or after transformation, by SV40 virus. Their findings suggest AKR cells carry the full RNA tumor virus genome in an unexpressed form.

Weiss et al. (42) induced avian tumor virus formation in normal chicken cells after treatment with ionizing radiation, chemical carcinogens and mutagens.

Spontaneous and chemical activation of C-type RNA viruses has been reported in cloned mouse cells (42, 43), rat (44, 45), cat (47-49), human tumor and normal human fibroblasts (50-52).

In certain instances, the virus isolates were oncogenic (53) and provided "helper" function in defective virus studies and possessed physical properties of the C-type RNA viruses (52).

Based on the above evidence of activation of endogenous C-type RNA viruses and viral gene expression in mammalian and avian cells, we determined whether treatment of normal human cells by hydrazine compounds and polycyclic hydrocarbons results in partial or complete expression of an endogenous human RNA virus. Detection of RNA dependent DNA polymerase activity (Reverse Transcriptase) and transmission electron microscopy (TEM) of fixed cell pellets were used to monitor expression.

Cell Line Used in this Study. The rationale for using the Detroit 550 (D550) (male, human foreskin fibroblast) cell strain (American Type Culture Collection CCL 109) was described in FY 77 Annual Report, based on our previous work with this cell line in SV40 studies.

HZ, MMH, UDMH, SDMH, ANA, PANA, PBNA and BP were assayed as cocarcinogens in the ST-FeSV-D550 cell virus system to determine if the interaction of these compounds affect virus transformation in a predictable manner.
V. Scientific Progress on Milestones Attained in FY 79

1. Tested ANA, PANA, PBNA, HZ, MMMH, UDMH and SDMH co-carcinogenesis assay system.

2. Determined dose and time relationships for napthylamines and hydrazines in co-carcinogenesis assay.


4. Endogenous virus activation did not occur in short-term (60-day) exposure of normal cells to HZ, MMMH, UDMH and SDMH.

VI. Final Report Summary (Segment II)

A. Results to Date (Segment II) on Virus Induced Neoplastic Transformation

1. Co-carcinogenesis studies

   Cells, Chemical Preparation and Co-carcinogenesis Assays. Human foreskin fibroblast cells (Detroit 550-CCL109, American Type Culture Collection, Rockville, Md.) were grown in EMEM (Gibco, Grand Island, N.Y.) supplemented with 1 mM sodium pyruvate, 1X non-essential amino acids, 2 mM glutamine, 50 μg gentamycin per ml (Schering Diagnostics, Port Reading, N.J.), 0.11% sodium bicarbonate, and 10% FBS (Sterile Systems, Ogden, Utah). Cells were serially passaged every 3 to 4 days at a 1:2 ratio and incubated at 37°C in 5% CO₂ atmosphere.

   Just prior to use, the ANA, PANA or PBNA were weighed and dissolved in spectral grade acetone at 10 mg/ml and a stock solution made by adding 1 ml to 100 ml pre-warmed (37°C) E-MEM + 10 FBS at pH 7.0. HZ MMMH or UDMH were pipetted directly into E-MEM + FBS, pH 7.0 at the desired working concentration (PPM). SDMH was dissolved in 0.1 N HCL
at 10 mg/ml and diluted in E-MEM + FBS to experimental concentrations. Stock cell cultures were trypsinized and seeded into 35 mm wells at a concentration of \(1 \times 10^5\) cells/well in 4.0 ml E-MEM + 10% FBS and incubated 18 hours prior to treatment. Cells pre-treated with chemicals prior to virus infection were incubated with designated concentrations of chemical for 90 minutes at the following time periods: 24 hrs, 6 hrs and 2 hrs, washed and treated with 1.0 ml of DEAE-dextran (40 \(\mu\)g/ml) in serum-free E-MEM. After 20 minutes, the cells were rinsed with E-MEM + 5% FBS, infected with 0.2 ml per well with each of four 2-fold virus dilutions and allowed to adsorb for 2 hours. Four wells were used per dilution of virus. The plates were rocked at 10 to 15 minute intervals to maintain an even distribution of inoculum and after adsorption, the inoculum was removed and replaced with 4 ml of growth medium. Cells post-treated with chemicals after virus infection were incubated with designated concentrations of chemicals 2, 6 or 24 hours for 90 minutes after virus adsorption. The medium was removed, washed and refed with growth medium. Cells were refed with fresh growth medium only on the 6th day after infection and subsequently fixed with buffered formalin and stained with Giemsa 3 to 4 days later. Foci appear as discrete areas consisting of round, hyper-refractile, enlarged fibroblast cells (10). These foci were counted at 25 to 40 X with a dissecting microscope.

In other experiments, the concentrations of chemicals used in these experiments were determined by treating non-virus infected cells for 90' to discern the appropriate concentration to use in the virus experiments. Cells were washed, refed and incubated 9-10 days, fixed in buffered formalin, stained with Giemsa and clones containing at least 50 cells were counted. Toxicity (surviving fraction) was determined by dividing
the average number of clones in treated wells by the average number of clones in control wells.

Virus-induced foci were counted in nontreated and chemically treated wells. FFU ± S.D. were determined for each treatment time and significance determined by Student's test. The figures in the text where percent inhibition is presented was determined by:

\[
\text{Percent inhibition} = \frac{\text{FFU chemically treated}}{\text{FFU control}} \times 100
\]

(a) Time and Dose Related Effects of ANA on ST-FeSV Transformation

Virus-directed transformation was significantly inhibited by chemical treatment (10 μg and 0.01 μg/ml) when cells were treated from 6 hours to 2 hours before virus infection, but not when treated 24 hours before infection. Furthermore, in virus infected cells treated with ANA, inhibition of transformation occurred at 2 hours post-infection (p.i.) but not at 6 or 24 hours p.i. (Fig. 1).

(b) Time and Dose-Related Effects of PANA on ST-FeSV Transformation

Three concentrations (20 μg/ml, 10 μg/ml, 0.1 μg/ml) inhibited ST-FeSV transformation in human cells. Inhibition was dose dependent and time-independent in that concentrations of 20 and 10 μg/ml significantly inhibited transformation at all 6 time periods tested where the lowest concentration (0.1 μg/ml) had no effect on virus directed transformation at any of the 6 time periods used in this test (Fig. 2).

(c) Dose- and Time-Related Effects of PBNA on ST-FeSV Transformation

Four (4) concentrations of PBNA were assayed: (20, 10, 0.1 and 0.01 μg/ml). The highest concentrations (20 and 10 μg/ml) significantly inhibited transformation at all 6 time periods with the greatest inhibitory effect seen when cells were treated before virus infection (Fig. 3). Maximum inhibition resulted (71% and 60%) when cells were
exposed to 10 μg/ml, 6 hrs before infection and 20 μg/ml, 24 hours before infection, respectively. Lesser concentrations (0.1 and 0.01 μg/ml) enhanced transformation at 6 hours p.i.

Thus, at the 2 higher concentrations of PBNA the effect on virus transformation was inhibitory while at the 2 lower concentrations, the predominant effect was enhancement of transformation.

(d) MMI4 (100 ppm and 10 ppm) significantly enhanced virus transformation in a dose-dependent manner when cells were exposed 2 hrs pre-infection (Fig. 4). Conversely, treatment of virus-infected cells resulted in significant inhibition of transformation when cells were treated 2 hrs or 6 hrs p.i. No effect was noted at 24 hrs p.i.

(e) SDMH (100 μg/ml and 10 μg/ml) likewise enhanced virus transformation when cells were treated 2 hrs pre-infection (Fig. 5). 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 p.i., but not at 6 or 24 hrs p.i.

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

(g) The results of HZ (60 ppm and 6 ppm) exposure are shown in Fig. 7. Fig. 7 is a composite figure of 3 separate experiments showing HZ effects on virus transformation. Like SDMH and MMI, cells exposed to HZ 2 hrs pre-infection, significantly enhanced virus transformation whereas exposure 2 hrs post-virus infection significantly inhibited transforming effect of the sarcoma virus as did MMI, SDMH, and UDNIH.
2. A. **Significance of Co-Carcinogenesis Studies in Relation to In Vitro Chemical Transformation and Neoplastic Transformation** (from Segment I)

Correlation of the above mentioned parameters with inhibition of virus transformation show that the test chemicals which chemically transform human cells *in vitro* which, in turn, produce neoplastic growth when injected in athymic mice, significantly inhibit virus transformation (Table I). These results, when correlated with the data from Dr. Mllio's work, suggest the napthylamines are carcinogenic when tested by the parameters listed in Segment I and II. HZ and UDMH showed a positive correlation in 3 of 3 listed parameters while MMH and SDMH did not (1 of 3). This data suggest ANA, PANA, PBNA, HZ and UDMH are carcinogens while SDMH and MMH have co-carcinogenic activity.

B. **Endogenous Virus Activation**

The results of attempts to activate endogenous human virus from HZ, MMH, SDMH and UDMH treated cells are shown in Table 2. Cells were constantly treated for 60 days and supernatants monitored for presence of RNA-directed DNA polymerase activity (Reverse Transcriptase) as a measure of virus expression. At the end of the experiment, the cells were removed from the flasks, processed for electron microscopic analysis, and examined for the evidence of 'budding' C-type virus. The RDDP results indicated no virus was released and analysis of treated cells by TEM showed no virus release from treated or untreated cells (Table 3).

C. **Cytotoxic Analyses of Petroleum and Shale Oil Derived Fuels**

Petroleum derived (PD) or shale oil derived (SOD) JP5 or diesel fuel marine (DFM) fuels cytotoxic analyses are shown in Table 3. No significant difference in LD₅₀'s were detected for PD or SOD fuels. The LD₅₀
for SOD-JP5 and PD-JP5 were 102 ppm and 100 ppm respectively while SOD-DFM and PD-DFM were 87 ppm and 85 ppm respectively. LD$_{50}$ for JP10 fuel was 91 ppm while RJ5 was 19 ppm.

VII. Discussion

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

ANA, PANA, and PBNA showed 100% correlation with the 2 parameters, whereas MMH and SDMH showed activity in the co-carcinogenesis assays, but not in the carcinogenesis assays. HZ and UDMH, like the napthylamines, 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 subtoxic concentrations were used.

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 (54). The major effect on virus anti-carcinogenic 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 napthylamines in that maximum inhibition occurred when virus infected cells were exposed to the hydrazines 2 hrs post-infection, whereas inhibition was observed at all 6 time periods with PANA and PBNA and at 3 time periods with ANA.
HZ, MMH, UDMH, SDMH and PAN-A have shown mutagenic, teratogenic or carcinogenic properties depending upon the assay used (55-59). Thus, these chemicals interact with host cell transcriptional or translational processes. In previous studies we concluded the inhibitory (anti-carcinogenic) effect of benzo(a)pyrene, Aflatoxin B, or N-acetoxy-2 fluorenyl acetamide on virus transformation was not due to decreased cellular proliferation or virus synthesis (10,59). Further, the anti-carcinogenic effect was abrogated when cells were exposed >24 hours 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 (60,61) co-carcinogenic (62,63) or no effect (64,65) on transformation depending on the virus or chemical used in the experiments, whereas in vitro studies with rat or mouse cells showed synergism (66-68).

We previously reported anti-carcinogenic activity with 3 known carcinogens on FeSV transformation of human cells in vitro. A recent report by Rhim and Arnstein (69) described anti-carcinogenic activity of an oncogenic murine virus on chemical-induced transformation of canine cells.

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

VIII. Methods of Procedure in Segment II

1. Viruses

Snyder-Thelen Feline Sarcoma Virus (ST-FeSV) - Feline sarcoma virus containing tumor tissue was supplied by Dr. G. Thelen and has undergone
2-4 in vitro passages in our laboratory. Ten to 20% suspensions of minced tumor tissue in Leibowitz medium (L-15) were homogenized for 2 minutes and centrifuged at 2300 x g for 20 minutes. Supernatants were recentrifuged as before, and the resulting supernatant centrifuged at 18,000 x g for 10 minutes. Final supernatants were passed through 0.45 μ filters or placed over sucrose for further purification. In the latter case, supernatants were layered over 5 ml of 45% sucrose (density = 1.2 g/ml) and centrifuged at 40,000 x g for 3 hours. Resulting virus-containing bands were dialysed to remove sucrose, aliquoted and frozen at -85°C. Virus was also produced from feline embryo-infected cells.

2. Cells
(a) Human foreskin fibroblast cells (normal male) designated Detroit 550 (American Type Culture Collection, Rockville, Md.) were grown in minimum essential medium Earle's Salts (MEM(E)), supplemented with 1X non-essential amino-acids, 1 mM sodium pyruvate, 2 mM glutamine, 50 μg/ml Gentamycin and 10% heat-activated fetal bovine serum. For routine passage, cells were grown to confluency in 120 cm² prescription bottles, dispersed with 0.1% trypsin prepared in incomplete MEM and subcultured on a 1:2 basis. Cells used in transformation assays were between passage levels 15 through 25 (Phase II).

Feline Embryo Cells (FE) - Feline embryo cell cultures prepared from 30 day fetuses were used from passage 3-10 for growth and virus quantitation assays with FeSV. Embryos were finely minced and trypsinized for 45 minutes. Dispersed cells were washed in L-15 medium containing 15% fetal calf serum and seeded into 400 ml prescription bottles. Early passage cultures were frozen at 102 x 10⁶ cells/ml in 2 ml ampoules for constant supply of low passage cells.
3. Transformation Assays

   a. Snyder-Thellen Feline Sarcoma Virus - Detroit 550 cells were seeded at concentrations of 1-2 x 10^5 cells per 35 mm wells in 4 ml of growth medium. After incubation for 24 hours, cells were pre-treated with 1 ml of DEAE-D dextran at 40 μg/ml for 20 minutes at room temperature, and washed with MEM(E) containing 5% FBS. Monolayers were infected with 0.2 ml of appropriate virus dilutions and allowed to adsorb for 2 hours. Inoculum were then removed and replaced with 4 ml of MEM + 10 FBS. Infected cells were refed after 3-5 days. Foci of altered, hyper-refractile cells confined to discrete areas appear within 6-8 days. Foci were enumerated at 12-14 days by fixing with buffered formalin and staining with Giemsa.

4. Test Materials

   Naphtylamines were suspended in acetone (10 mg/ml), SDMH in 0.1 NHCl (10 mg/ml) and hydrazines in growth medium at 10^6 ppm and added to target cells at pre-determined dose levels. In previous studies in this laboratory with chemical carcinogens and mutagens, stock solutions of water-insoluble chemicals were dissolved in acetone at 10 mg/ml and a stock solution made by adding 1 ml to 100 ml prewarmed complete medium at pH 7.0 (10). Further dilutions were made in complete prewarmed medium to obtain the needed concentration. This procedure was used in this study where properties of test materials permit. Subtoxic dose levels were determined by cell-survival studies with target cells by comparison of cloning efficiencies of treated vs. untreated cells.

5. Electron Microscopy

   a. Thin section. Cells were removed from the glass either by trypsinization or scraping, washed in PBS and centrifuged at 150 x g for
10 minutes in an international centrifuge. Cell pellets were fixed with either 2% glutaraldehyde followed by 1% osmium tetroxide or Dalton's chrome osmium fixative (70). After dehydration in graded ethyl alcohol, the cells were embedded in an Epon araldite mixture by the procedure of Mollenhauer (71) and thin sectioned with glass knives on a Porter-Blum microtome. Sections were stained with 2% uranyl acetate and with lead citrate and examined with a Phillips Model 200 or 300 electron microscope (72).

b. Negative staining techniques. Equal volumes of suspected virus suspensions were thoroughly mixed with 0.5% phosphotungstic acid solution (PTA) containing 0.2% sucrose, pH 7.0. Carbon coated colloidal covered grids were dried and scanned with the electron microscope at a magnification of 30,000 X.

6. Reverse Transcriptase Enzyme Assay

20-30 ml of chemically treated virus infected tissue culture media were clarified at 10,000 g for 15 min at 4°C. Clarified supernatants were then centrifuged at 100,000 g for 90 min at 4°C to pelletize any virus present. Virus pellets were triturated in 50-80 μl of Tris-Triton suspension buffer (5 mM Tris-HCl at pH 8.1; 1 mM dithiothreitol (DTT); 0.1% Triton X-100 and 0.5 M KCl. 10 μl aliquots of the above virus suspension were incubated in a 37°C water bath for 60 min in a final volume of 50 μl containing: 5 μl of Poly(rA)ₙ (0.3 μg) premixed with 4 μl of Oligo(dT) 12-18 (0.4 μg) and 1 μl of H₂O; 5 μl of 50 mM Tris - 0.1% Triton X-100, 5 μl of 1 mM manganese acetate, 5 μl of 20 mM DTT, 5 μl of H₂O and 10 μl of (5 μCi) (³H) thymidine triphosphate (TTP) (spec. act. 17 Ci/mmol). Following incubation, 40 μl of the reaction mixture were spotted on Whatman 3 filter paper disks, washed 4 times with 500 ml cold
acid wash (10% sodium pyrophosphate, 0.7% hydrochloric acid and 5%
glacial acetic acid) and a final wash in 500 ml of a 1:1 mixture
of ethanol-ether for 10 min. The disks were dried and (3H) TTP incor-
poration measured by scintillation counting the cold acid precipitable
material in 10 ml scintillation cocktail composed of 15.2 g BBOT in
1 gal toluene.

IX. Support Data

1. 16 copies - "Induction of Retrovirus Non-Producer Human Cells to
Producer Cells by Dexamethasone". By: JR Blakeslee, A Elliot
and D Turner

2. 16 copies - "Factors Affecting Feline Retrovirus Infectivity and
Oncogenicity." In: Feline Leukemia, Ed. RG Olsen, CRC Press,
Table 1. Correlation between Inhibition of Virus Transformation, *In Vitro* Chemical Transformation and Neoplastic Transformation

<table>
<thead>
<tr>
<th>Chemical</th>
<th><em>In Vitro</em> Chemical Transformation</th>
<th>Neoplastic Transformation</th>
<th>Inhibition of Virus Transformation</th>
</tr>
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<td>ANA</td>
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</tr>
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<td>PSNA</td>
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<tr>
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</tr>
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<tr>
<td>SDMH</td>
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</table>

*BP* - Benzo(a)pyrene  
**Pyr. - Pyrene
Table 2. RDDP Activity in Cell Supernatants from HZ, MMH, SDMH and UDMH Treated Cells

**EXPERIMENT 1**

<table>
<thead>
<tr>
<th>Day Post-Treatment</th>
<th>Day Post-Treatment</th>
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<th>MMH (80 PPM)</th>
<th>UDMH (100 PPM)</th>
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</thead>
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<tr>
<td>(Untreated)</td>
<td>(CPM x 10^-3 of 3H)</td>
<td>(CPM x 10^-3 of 3H)</td>
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<td>2.6</td>
<td>1.6</td>
</tr>
<tr>
<td>27</td>
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<td>1.3</td>
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<td>1.3</td>
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<td>1.9</td>
<td>1.5</td>
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<td>32</td>
<td>32</td>
<td>2.7</td>
<td>2.2</td>
<td>3.5</td>
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<tr>
<td>36</td>
<td>36</td>
<td>N.D.*</td>
<td>3.0</td>
<td>2.6</td>
</tr>
<tr>
<td>40</td>
<td>40</td>
<td>1.5</td>
<td>2.2</td>
<td>1.9</td>
</tr>
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<td>45</td>
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<td>2.4</td>
<td>2.5</td>
<td>2.2</td>
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<tr>
<td>52</td>
<td>52</td>
<td>1.8</td>
<td>2.1</td>
<td>2.4</td>
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<tr>
<td>60</td>
<td>60</td>
<td>2.0</td>
<td>1.8</td>
<td>2.1</td>
</tr>
</tbody>
</table>

FeLV Standard CPM = 96.6

Background - CPM = 0.029

* = N.D. = Not Determined

**EXPERIMENT 2**

<table>
<thead>
<tr>
<th>Days Post-Treatment</th>
<th>Untreated</th>
<th>SDMH (100 µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPM x 10^-3</td>
<td>CPM x 10^-3 of 3H</td>
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<td>5</td>
<td>1.5</td>
<td>1.8</td>
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<td>10</td>
<td>1.8</td>
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<td>14</td>
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<td>1.6</td>
<td>1.4</td>
</tr>
<tr>
<td>59</td>
<td>1.5</td>
<td>1.2</td>
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</table>

FeLV Standard CPM = 50.3

Background - CPM = 0.025
Table 3. SEM Results from Cells Exposed to HZ, MMH and UDHH

<table>
<thead>
<tr>
<th>Chemical</th>
<th># virus positive</th>
<th># grids examined</th>
</tr>
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<tbody>
<tr>
<td>Hydrazine</td>
<td>0/10</td>
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<tr>
<td>Monomethyl hydrazine</td>
<td>0/4</td>
<td></td>
</tr>
<tr>
<td>Unsymmetrical dimethylhydrazine</td>
<td>0/7</td>
<td></td>
</tr>
<tr>
<td>Symmetrical dimethylhydrazine</td>
<td>0/5</td>
<td></td>
</tr>
</tbody>
</table>

*As a positive control, F422 cells, a canine thymocyte cell line which constantly sheds C-type feline leukemia virus, were prepared in conjunction with the chemically treated cells to control the fixation procedure and to provide magnification and morphological parameters.
Table 4: LD$_{50}$ cytotoxicity of Shale Oil and Petroleum Derived Fuels in HSF Cells

<table>
<thead>
<tr>
<th>Fuel</th>
<th>Derived From</th>
<th>LD$_{50}$ (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JP5</td>
<td>Shale</td>
<td>102</td>
</tr>
<tr>
<td>JP5</td>
<td>Petroleum</td>
<td>100</td>
</tr>
<tr>
<td>DFM$^1$</td>
<td>Shale</td>
<td>85</td>
</tr>
<tr>
<td>DFM</td>
<td>Petroleum</td>
<td>87</td>
</tr>
<tr>
<td>RJ5</td>
<td>Petroleum</td>
<td>19</td>
</tr>
<tr>
<td>JP10</td>
<td>Petroleum</td>
<td>91</td>
</tr>
</tbody>
</table>

$^1$ 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.
Figure 1

CONCENTRATION

- 10μg/ml
- 0.01μg/ml
- 0.001μg/ml

* Significance 0.050 - 0.001

UNTREATED CONTROLS

UNTREATED CONTROLS

TREATMENT TIME IN RELATION TO VIRUS INFECTION (HOURS)

FOLD ENHANCMNT

FOLD INHIBITION

-6 -2 +2 +6 +24

5 4 3 2 1 0 1 2 3 4 5 100
Fig. 2. Effect of PANA on ST-FeSV transformation. Cells treated with PANA as described in Fig. 1.
Fig. 3. Effect of PBNA on ST-FeSV transformation. Cells treated with PBNA as described in Fig. 1.
Figure 3

[Graph showing concentration of virus in relation to treatment time and fold enhancement/inhibition. Legend includes symbols for concentrations (10 ng/mL, 0.1 ng/mL) and significance levels (0.005 - 0.001).]
Fig. 4. Effect of MMH on ST-FeSV transformation. Cells treated with MMH as described in Fig. 1.
Fig. 5. Effect of SDMH on ST-FeSV transformation. Cells treated with SDMH as described in Fig. 1.
The graph illustrates the effect of virus infection on transformation over time. Two concentrations of sym-dimethylhydrazine are shown: 100 μg/ml and 10 μg/ml. Asterisks indicate significant differences. The x-axis represents time in hours (from -24 to +24), and the y-axis represents the effect on transformation.
Fig. 6. Effect of UDMH on ST-FeSV transformation. Cells treated with UDMH as described in Fig. 1.
A graph showing the effect on transformation over treatment time (hours). The x-axis represents treatment time in hours, ranging from -24 to +24. The y-axis represents the effect on transformation, ranging from 0.2 to 1.4.

- **100 PPM-unsym. dimethylhydrazine** represented by filled circles.
- **10 PPM-unsym. dimethylhydrazine** represented by filled triangles.
- An asterisk indicates a significant effect.

An arrow labeled "virus infection" points to the positive effect at time 0. The graph shows fluctuations in effect over time, with notable peaks at certain time points.
Fig. 7. Effect of HZ on ST-FeSV transformation. Cells treated with HZ as described in Fig. 1.
Effect on Transformation


Cover Sheet
for
Chapter 6, Authors, M.J. Tarr and J.R. Blakeslee entitled:

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Chapter 2:
Factors Affecting Feline Retrovirus Infectivity and Oncogenicity

Melinda J. Tarr
James R. Blakeslee

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Chapter 3
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 Alhstrom 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 addition 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 addition 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 Methyl nitrosourea: 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. Methyl nitrosourea (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 cat's 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 reticuloendothelial 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
<table>
<thead>
<tr>
<th>Site</th>
<th>Environment</th>
<th>Reaction</th>
<th>Product</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site 1</td>
<td>Exposed</td>
<td>Reaction A</td>
<td>Product X</td>
<td>50%</td>
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<tr>
<td>Site 2</td>
<td>Control</td>
<td>Reaction B</td>
<td>Product Y</td>
<td>30%</td>
</tr>
</tbody>
</table>

**Notes:**
- Site 1 exposure caused a 20% increase in reaction rate.
- Further experiments are needed to confirm the yield predictions.

**References:**
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 10, 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 Lovey (25) provided evidence that for xenotropic and the FAIR 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 17B 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.  

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Chrysotile</th>
<th>Crocidolite</th>
<th>Amosite</th>
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</thead>
<tbody>
<tr>
<td>Base Composition</td>
<td>Hydrate magnesium silicate</td>
<td>Hydrated silicate of iron and sodium</td>
<td>Hydrated silicate of iron and magnesium</td>
</tr>
<tr>
<td>Texture of fiber</td>
<td>Silky; soft</td>
<td>Harsh</td>
<td>Coarse</td>
</tr>
<tr>
<td>Flexibility and Spinning Properties</td>
<td>Excellent</td>
<td>Fair</td>
<td>Poor</td>
</tr>
<tr>
<td>Major Properties</td>
<td>Flexible heat resistant</td>
<td>Flexible heat resistant</td>
<td>Brittle</td>
</tr>
</tbody>
</table>

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/mm² 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 preceding 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 $^3\text{H}$ 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 3–12 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.
REFERENCES


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.

<table>
<thead>
<tr>
<th>Cat No.</th>
<th>Rx</th>
<th>FeLV Viremia</th>
<th>Highest FOCMA of titer</th>
<th>LSA disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>597</td>
<td>FeLV</td>
<td>-</td>
<td>16</td>
<td>-</td>
</tr>
<tr>
<td>728</td>
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<td>32</td>
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<td>729</td>
<td>-</td>
<td>32</td>
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<td>-</td>
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<tr>
<td>735</td>
<td>-</td>
<td>-</td>
<td>64</td>
<td>-</td>
</tr>
<tr>
<td>743</td>
<td>-</td>
<td>64</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>744</td>
<td>-</td>
<td>16</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>672</td>
<td>FeLV + MNU</td>
<td>+</td>
<td>4</td>
<td>+</td>
</tr>
<tr>
<td>676</td>
<td>+</td>
<td>4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>701</td>
<td>+</td>
<td>4</td>
<td>+</td>
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<td>703</td>
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<td>64</td>
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<tr>
<td>706</td>
<td>+</td>
<td>4</td>
<td>+</td>
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<tr>
<td>707</td>
<td>+</td>
<td>4</td>
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<tr>
<td>718</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>734</td>
<td>-</td>
<td>128</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 2. Proportion of Cats Developing Viremia, FOCMA Antibody, or LSA Disease Following Exposure to MNU and/or FeLV.

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

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.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>736</td>
<td>34d</td>
<td>747</td>
<td>15d</td>
</tr>
<tr>
<td>800</td>
<td>84d</td>
<td>813</td>
<td>17d</td>
</tr>
<tr>
<td>802</td>
<td>45d</td>
<td>814</td>
<td>17d</td>
</tr>
<tr>
<td>803</td>
<td>84d</td>
<td>819</td>
<td>16d</td>
</tr>
<tr>
<td>mean</td>
<td>74.25</td>
<td>mean</td>
<td>16.25d</td>
</tr>
</tbody>
</table>
Figure 1. Cutaneous allografts in MNU-treated and control cats.

A) Cat number 300B, 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.
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).
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.
Figure 4. Lymphocyte blast transformation response of MNU-treated and control cats to KLH following KLH immunization. Vertical bars represent standard error of the mean (n = 4).
CORRECTED STIMULATION INDEX

-○- MNU treated cats
-●- untreated control cats

WEEKS

- MNU
- KLH

1 2 3 4 5 6 7 8 9 10 11 12
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).
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).
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 X 10⁵) 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 adsorption for 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:

\[
\frac{\text{FFU chemically treated}}{\text{FFU control}} \times 100
\]

Significance was determined by Student's t-test. Concentrations of BP used were: Δ, 15.0 μg/ml; 0, 5.0 μg/ml; △, 1.0 μg/ml; *, significant inhibition.

BENZOD(c)PYRENE AND STFeSV FOCUS FORMATION

\[ \Delta - 15.0 \mu g/ml \]
\[ O - 5.0 \mu g/ml \]
\[ \triangle - 1.0 \mu g/ml \]
\[ \ast - \text{SIGNIFICANT INHIBITION} \]

TREATMENT TIME IN RELATION TO VIRUS INFECTION (hrs)

FOLD-ENHANCEMENT

% INHIBITION
Figure 9. Inhibition of ST FeSV transformation by N-acetolyl-2-fluorenlyl Acetamide.

D350 cells (1 X 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.

N-ACETOXY 2-FLUORENYL ACETAMIDE AND STFeSV FOCUS FORMATION

Δ = 1.0 μg/ml
○ = 0.1 μg/ml
* = SIGNIFICANT INHIBITION

CONTROL

FOLD-ENHANCEMENT

% INHIBITION

-24 -6 -2 0 +2 +6 +24
TREATMENT TIME IN RELATION TO VIRUS INFECTION (hrs)
Figure 10. Inhibition of ST FeSV transformation by aflatoxin B1.

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

AFLATOXIN B1 AND STFeSV FOCUS FORMATION

\[ \Delta - 1.0 \mu g/ml \]
\[ \circ - 0.1 \mu g/ml \]
\[ * - \text{SIGNIFICANT INHIBITION} \]

TREATMENT TIME IN RELATION TO VIRUS INFECTION (hrs)
Legend Table 4:

Detroit 550 cells (1.5 X 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 from infected 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.
Table 4: Methods of Cellular Harvest and DXM Treatment: Effect on Virus Synthesis.

<table>
<thead>
<tr>
<th>Method of Harvest</th>
<th>FFU/ml (-)DXM</th>
<th>Fold Increase</th>
<th>FFU/ml (+)DXM</th>
<th>Fold Increase</th>
<th>RT Assay (-)DXM CPM</th>
<th>RT Assay (+)DXM CPM</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells scraped</td>
<td>234</td>
<td>87X</td>
<td>30,325</td>
<td>9X</td>
<td>8,848</td>
<td>77,427</td>
<td></td>
</tr>
<tr>
<td>Cells trypsinized</td>
<td>0</td>
<td>(-)</td>
<td>192</td>
<td>12X</td>
<td>8,464</td>
<td>102,574</td>
<td></td>
</tr>
<tr>
<td>Cells trypsinized</td>
<td>233</td>
<td>100X</td>
<td>23,300</td>
<td>11X</td>
<td>23,255</td>
<td>148,413</td>
<td></td>
</tr>
<tr>
<td>CF Only(d)</td>
<td>349</td>
<td>56X</td>
<td>19,125</td>
<td></td>
<td>22,462</td>
<td>241,903</td>
<td></td>
</tr>
</tbody>
</table>

* - CF = Culture fluids.
Cells ($1 \times 10^5$) 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:

\[
\text{Percentage inhibition} = \left( \frac{\text{FFU asbestos treated}}{\text{FFU virus control}} \right) 
\]

Fold enhancement determined by:

\[
\text{Fold enhancement} = \left( \frac{\text{FFU asbestos treated}}{\text{FFU virus control}} \right)
\]

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

- $0.0001 \mu g/ml$
- $1.0 \mu g/ml$
- $10.0 \mu g/ml$
- *SIGNIFICANCE = 0.001–0.025*

PERCENT INHIBITION

FOLD-ENHANCEMENT

UNTREATED CONTROLS

TREATMENT TIME IN RELATION TO VIRUS INFECTION (HOURS)
Figure 12. Time and dose related effect of Chrysotile asbestos on St FeSV transformation. Cells were treated with Chrysotile asbestos as described in Figure 11.
CONCENTRATION

- 0.0001 μg/ml
- 0.001 μg/ml
- 0.01 μg/ml
- 0.1 μg/ml
- SIGNIFICANCE - 0.001 - 0.025

FOLD INHIBITION

FOLD ENHANCEMENT

UNTREATED CONTROL

TREATMENT TIME IN RELATION TO VIRUS INFECTION (HOURS)
Figure 13. Inhibition of St FeSV transformation by Amosite asbestos. Cells were treated with Amosite asbestos as described in Figure 11.
CONCENTRATION

- 0.0001 μg/ml
- 0.001 μg/ml
* - SIGNIFICANCE 0.001 - 0.025

UNTREATED CONTROL

PERCENT INHIBITION

FOLD-ENHANCEMENT

TREATMENT TIME IN RELATION TO VIRUS INFECTION (HOURS)
Table 6. Pre-incubation of ST FeSV with Asbestos. Effect on Transformation.

<table>
<thead>
<tr>
<th>Asbestos</th>
<th>Concentration µg/ml</th>
<th>Transformed Foci FFU/0.2 ml X 10-2</th>
<th>Effect on Transformation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Treated</td>
<td>Control</td>
</tr>
<tr>
<td>Amonosite</td>
<td>10</td>
<td>9.0 ± 2.6</td>
<td>22.5 ± 4.1</td>
</tr>
<tr>
<td>Chrysotile</td>
<td>10</td>
<td>24.5 ± 4.2</td>
<td>22.5 ± 4.1</td>
</tr>
<tr>
<td>Crocidolite</td>
<td>-10</td>
<td>23.5 ± 1.4</td>
<td>22.5 ± 4.1</td>
</tr>
</tbody>
</table>

(a) = FFU ± S.D.

(b) = (p) significance determined by Student's t-test.

1 = 1.0 ml stock FeSV (1.0 X 10⁵ FFU/ml) was incubated 30' at 30°C with 1.0 ml HUSS containing 10 µg of asbestos. Asbestos was removed by centrifugation, 2000 rpm for 15' at 4°C. Supernatants were aspirated and remaining virus titrated in D550 cells.
Table 7. Concomitant Treatment with Amosite, Chrysotile or Crocidolite Asbestos with Snyder-Theilen Feline Sarcoma Virus Infection.

<table>
<thead>
<tr>
<th>Asbestos</th>
<th>Concentration (μg/ml)</th>
<th>Transformed Foci FFU/0.2 ml x 10^-2</th>
<th>Effect on Transformation (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amosite</td>
<td>10</td>
<td>Treated: 51.9±4.0(a) Control: 22.5±4.1</td>
<td>2.3↑ 0.001(b)</td>
</tr>
<tr>
<td>Chrysotile</td>
<td>10</td>
<td>Treated: 46.4±4.8 Control: 22.5±4.1</td>
<td>2.1↑ 0.001</td>
</tr>
<tr>
<td>Crocidolite</td>
<td>10</td>
<td>Treated: 42.5±12.1 Control: 22.5±4.1</td>
<td>1.9↑ 0.050</td>
</tr>
</tbody>
</table>

(a) FFU = Focus Forming Units ± S.D.
(b) (p) = significance determined by Student's t-test.
CONCENTRATION

Δ - 0.0001 μg/ml
○ - 1.0 μg/ml
■ - 10.0 μg/ml
* - SIGNIFICANCE - 0.001 - 0.025

TREATMENT TIME IN RELATION TO VIRUS INFECTION (HOURS)

FOLD-ENHANCEMENT

PERCENT INHIBITION

UNTREATED CONTROLS

TREATMENT TIME IN RELATION TO VIRUS INFECTION (HOURS)

FOLD 10.5

PERCENT INHIBITION

UNTREATED CONTROL
N-ACETOXY 2-FLUORENYL ACETAMIDE AND STFeSV FOCUS FORMATION

△ - 1.0μg/ml
○ - 0.1μg/ml
* - SIGNIFICANT INHIBITION

CONTROL

TREATMENT TIME IN RELATION TO VIRUS INFECTION (hrs)

AFLATOXIN B1 AND STFeSV FOCUS FORMATION

△ - 1.0μg/ml
○ - 0.1μg/ml
* - SIGNIFICANT INHIBITION

CONTROL

TREATMENT TIME IN RELATION TO VIRUS INFECTION (hrs)
Ave. grains / nucleus

Ave. grains / nucleus

Time (Hours)

Time (Hours)
BENZO(a)PYRENE AND STFeSV FOCUS FORMATION

- Δ - 15.0μg/ml
- ○ - 5.0μg/ml
- ▲ - 1.0μg/ml
- * - SIGNIFICANT INHIBITION

TREATMENT TIME IN RELATION TO VIRUS INFECTION (hrs)

% INHIBITION

FOLD-ENHANCEMENT
Figure 14. Excision Repair in Feline Fibroblasts from Different Aged Cats.

Feline fibroblast cells were grown from biopsied skin and used at passage 3. Cells were seeded on 1 x 3 sterile microscope slides and incubated 2 days at 37°C. Cells were washed in HBSS and incubated in Arginine deficient medium (ADM) plus dialyzed fetal bovine serum for 2 days. ADM containing 2 mm hydroxyurca was added to cells and incubated 1 h. Cells were washed and exposed to oVv light source at 100 ergs/mm². Cells were refeed with ADM-HU medium containing 0.5 uCi ³H-thymidine per ml and incubated at 37°C at 2, 6, 12, 18 and 24 hour intervals. Two control and 2 test slides were removed for fixation in methanol:acetic acid, rinsed 3X in 95% ethanol and air dried. In dark room, slides were dipped in 45°C Kodak NTB2 nuclear track emulsion and dried for 20 minutes. Slides were placed in light-tight box with a dessicant and incubated 4-7 days at 4°C. Slides were developed with D-19 Kodak developer and stained with H and E. Fifth cells per slide were counted and averaged and background grain counts subtracted.
INDUCTION OF RETROVIRUS NON-PRODUCER HUMAN CELLS TO PRODUCER CELLS BY DEXAMETHASONE

JAMES BLAKESLEE, ANN ELLIOT AND DEBRA TURNER.
Department of Veterinary Pathobiology, 1923 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. 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 FeSV6 infected Detroit 350 neonatal human skin fibroblast cells produced very low levels of transforming virus when supernatant fluids from these cultures were titrated in normal D350 cells. The addition of 1.0 μg/ml DXM (9α-Fluoro-6β-methyl prednisolone) 24 hours post infection, significantly increased both FeSV and FeLV 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 X 10^6 FFU/ml in DXM treated cultures, a 2.6 fold excess when compared to FeSV.

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

GSA and FOCMA were detected in both FeSV infected DXM treated cells and FeSV infected cells only. Uninfected-cells, cell treated with 0.2% acetone, and/or DXM were negative.
TABLE I

DXM ENHANCED ST-FeSV AND FeLV SYNTHESIS IN D550 CELLS

<table>
<thead>
<tr>
<th>Virus and Exp #</th>
<th>Virus Titer (μg/ml X 10^-2)</th>
<th>Fold Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(-) DXM</td>
<td>(+) DXM</td>
</tr>
<tr>
<td>(1)</td>
<td>2.3</td>
<td>203.2</td>
</tr>
<tr>
<td>(2)</td>
<td>2.3</td>
<td>233.0</td>
</tr>
<tr>
<td>(3)</td>
<td>3.4</td>
<td>191.2</td>
</tr>
<tr>
<td>Untreated Control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>FeLV</td>
<td>mean - 2.7 ± .5</td>
<td>mean - 209.2 ±17.6</td>
</tr>
<tr>
<td></td>
<td>600.00</td>
<td></td>
</tr>
</tbody>
</table>

* = Determined by Student's "t" test.

Wu et al. 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. 8,9

ACKNOWLEDGEMENTS

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REFERENCES

4. Schaller et al. (1976), Cancer Res. 36, 1880-1887.
5. Heding et al. (1976), Cancer Res. 36, 1647-1652.