Endemic Pneumonia Virus Isolated from Malignant Lymphoma Induced in Swiss Mice by 9,10-Dimethyl-1,2-benzanthracene

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SUMMARY

Extracts of malignant lymphomas induced by giving injections to newborn Swiss mice of 9,10-dimethyl-1,2-benzanthracene (DMBA) have been placed in contact with mouse embryo tissue cultures before inoculation into other newborn mice and hamsters, in an effort to demonstrate possible viral etiology of the tumor. Extracts of eight primary lymphomas and one transplanted lymphoma have all yielded a virus in tissue culture characterized by a destructive cytopathogenic effect (CPE). No similar destructive CPE was produced by extracts of thymus, spleen, liver, or kidney of normal Swiss mice, but extracts of lung caused a similar CPE to that produced by the lymphoma extracts. When culture medium containing the virus from DMBA-induced lymphomas was inoculated into newborn Swiss mice no difference in tumor incidence was observed between these mice and groups given inoculations of medium from spleen extract-treated cultures and groups left untreated. One hamster of a group of 31 given inoculations, when newborn, of the virus developed a malignant lymphosarcoma at 4 months of age; but this is not considered to be due to the virus. Cell-free extracts of this lymphosarcoma inoculated into newborn hamsters did not result in the development of tumors of any kind during a period of 1 year.

Both mice and hamsters given inoculations of the virus developed an incidence of typical endemic pneumonia lesions which was significantly higher than normal. The lesions were of greater severity than usually seen in untreated mice and frequently resulted in the death of the animal. Some biological properties of the virus are reported.

There are a few reports in the literature in which authors claim to have shown tumor virus activity in tumors induced by chemical carcinogens (13, 14, 16). Peacock (17) has criticized the work of McIntosh and Selbie (14) on the basis of morphological changes in the tumors which became filtrable and the fact that in some cases the filtrable tumor arose at a site distant from the implantation of the carcinogen. He concludes that only one of these tumors could be accepted as a virus-transmissible sarcoma derived from a tar sarcoma of apparently similar histology. The case reported by Oberling and Guerin (16) was a spindle-cell sarcoma arising in the leg of a chicken 32 months after the injection of methylcholanthrene at the same site. This tumor was transplantable, and tests for virus activity were positive at the fifth and sixth passages but not in earlier or later passages. Since no other virus-induced tumor was maintained at the laboratory at the time when these experiments were done it has been generally accepted that this was a true example of a chemically induced tumor which showed transient evidence of a virus etiology.

The demonstration by Pietra et al. (18) that malignant lymphomas could be induced in Swiss mice within 11–24 weeks by the injection of a small dose of 9,10-dimethyl-1,2-benzanthracene into newborn animals offered an opportunity to investigate the possible role of virus in the induction of this lymphoma. The experiments reported here, with some of the newer technics for demonstrating virus activity such as the inoculation of
newborn hosts and growth in tissue culture, have failed to show any evidence of tumor virus activity in DMBA-induced lymphoma but have shown that a latent virus infection in the mice may localize in tumor tissue and complicate the results of such work. Some of this work has been reported elsewhere (2).

MATERIALS AND METHODS

Animals used in these experiments were the Connaught strain of Swiss mice (Connaught Medical Research Laboratories, University of Toronto) and the Syrian hamster (High Oak Ranch, Richmond Hill, Ontario). Lymphomas were induced by the subcutaneous injection of approximately 60 μg of 9,10-dimethyl-1,2-benzanthracene (DMBA) in .02 ml. of 1 per cent gelatin into the interscapular region of newborn mice less than 24 hours old. The DMBA suspension was prepared according to the method of Pietra et al. (18).

Preparation of tissue extracts.—Lymphomas were found in moribund animals as early as 70 days and as late as 170 days after injection of DMBA. Post-mortem examination showed large tissue masses arising from the thymus and extending over the heart and lungs. Microscopically the tissue consisted of undifferentiated cells with little or no normal thymus remaining. Lung adenomas were often present concurrently, but the animals were not examined in detail for the presence of other tumors. Extracts of the lymphomas or tissues from normal animals were made by homogenizing the tissues in a Virtis “45” homogenizer in 10 volumes of sterile saline for 1 minute. The homogenate was centrifuged at 1500 × g for 15 minutes to remove coarse debris. The supernatant was subjected to 2 cycles of centrifugation at approximately 8000 × g for 1 hour in a swingout rotor at 4°C. The resulting clear supernatant was frozen and stored in a commercial deep freeze.

Mouse embryo cultures.—Cultures were prepared from normal Swiss embryos with 0.25 per cent trypsin in Hanks balanced salt solution without calcium or magnesium. The cells obtained from the first trypsinization were discarded, and those from a second trypsinization used for seeding 2-ounce bottles with approximately 2 × 10⁶ cells per bottle. The growth medium generally consisted of 30 per cent 1066 synthetic medium (Connaught Medical Research Laboratories, University of Toronto) and 20 per cent calf serum plus penicillin and streptomycin. In some experiments the medium consisted of 50 per cent 1066, 50 per cent Hanks balanced salt solution, and 20 per cent calf serum plus antibiotics. The cells grew equally well in either medium and within 2–3 days were almost complete monolayers. At this stage 1 ml. of the extracts to be tested was added to 9 ml. of growth medium bathing the cells, and the cultures examined daily for any changes. The culture medium was replaced twice a week with fresh medium containing no tissue extracts, and the old medium was frozen and stored in the deep freeze. The cells were left in the original culture bottles and allowed to peel off and reseed themselves, a procedure which took place several times during the observation period of up to 2 months if the cultures were not destroyed by viral activity. Serial passage of the culture medium was done by taking 1 ml. of old medium and mixing with 9 ml. of fresh. When it was desired to test culture media for possible viral activity in mice the frozen media were thawed, centrifuged at 1500 × g for 15 minutes at 4°C, and the supernatant was injected subcutaneously in the interscapular region of Swiss mice within 24 hours of birth.

RESULTS

Cytopathogenic activity of DMBA-induced lymphoma extracts for primary mouse embryo cultures. —Within 6–8 days after placing the original extract of the first lymphoma (#1) tested in contact with mouse embryo cells it became apparent that a marked cytopathogenic effect (CPE) was occurring. It began with the appearance of holes in the monolayer giving a “moth-eaten” effect. Rounded granular cells were present at the periphery of the holes. Very rapidly the monolayer disintegrated, leaving only scattered dying and dead cells (Figs. 1–3). Cultures usually did not recover after treatment with any of the nine different lymp-
phoma extracts tested, and when recovery occurred it did so only during the first and second passages of an extract.

Table 1 shows the history of twenty serial passages of extract #1, and it can be seen that the time required for the CPE to appear became shorter with succeeding passages until it remained steady at 1–3 days. No recovery occurred in any of the first and second passages which resulted in varying other six caused damage to the cultures during the extracts of eight different primary DMBA.

Phoma extracts tested, and when recovery occurred it did so only during the first and second passages of an extract. The plant generations, with tissue homogenates. It can extracts #1 (designated extract #9 in Table 2). This first transplant generation of the tumor used for induced lymphomas and a similar extract of the first transplant generation of the tumor used for extract #1 (designated extract #9 in Table 2). This tumor was carried successfully for three transpassages of an extract.

Table 2 summarizes the results obtained with extracts of eight different primary DMBA-induced lymphomas and a similar extract of the first transplant generation of the tumor used for extract #1 (designated extract #9 in Table 2). This tumor was carried successfully for three transplant generations, with tissue homogenates. It can be seen that three of the extracts completely destroyed the embryo cultures in all passages. The other six caused damage to the cultures during the first and second passages which resulted in varying degrees of peeling of the monolayer due to the sloughing off of damaged, granular cells. In some cases the cultures recovered and went on to form new monolayers, but this occurred only during the first two passages. Medium taken from the second passage while the damage was apparent resulted in complete destruction in subsequent passages, with the typical “moth-eaten” appearance followed by disintegration of the monolayer.

**Effect of extracts of normal tissues on primary mouse embryo cultures.**—Various tissues were removed for extraction purposes from freshly killed normal Swiss mice of approximately the same age as those bearing DMBA lymphomas. Each extract was made from the pooled tissues of five animals and was prepared in exactly the same way as those of the tumors. These extracts were tested on monolayers of mouse embryo cells for their capacity to cause a CPE (Table 3).

Spleen, liver, kidney, and thymus extracts never resulted in destruction of the embryo monolayers, even after six serial passages, although some damage—consisting of rounding and granulation of a small proportion of the cells—was evident in one of the spleen and one of the thymus extracts tested. This damage occurred during the first three passages but not in later ones. The medium for passage was taken during the height of the CPE when it was present or 3–6 days after exposure of the cultures to the extract under test.

**TABLE 2**

<table>
<thead>
<tr>
<th>Tumor extract</th>
<th>Passages</th>
<th>No. cultures</th>
<th>No. showing CPE</th>
<th>Days to CPE</th>
<th>No. recovered*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>20</td>
<td>134</td>
<td>134</td>
<td>1–16</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>7</td>
<td>41</td>
<td>30</td>
<td>2–34</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>43</td>
<td>27</td>
<td>2–21</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>39</td>
<td>34</td>
<td>3–32</td>
<td>5</td>
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<td>5</td>
<td>24</td>
<td>18</td>
<td>3–25</td>
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<td>7</td>
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<td>27</td>
<td>21</td>
<td>3–21</td>
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<td>5</td>
<td>24</td>
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<td>3–21</td>
<td>4</td>
</tr>
<tr>
<td>9†</td>
<td>5</td>
<td>15</td>
<td>15</td>
<td>3–13</td>
<td>0</td>
</tr>
</tbody>
</table>

* Recovery occurred in some cultures during 1st and 2d passages—never in later passages.
† Tumor extract #9 was made from a transplant of the tumor used for tumor extract #1.

Lung extracts did cause a CPE which resembled in every respect that produced by DMBA lymphosarcoma extracts. In every case the cultures were destroyed, with the exception of one bottle treated with the first passage of lung extract #1.

**Inoculation of culture media into newborn Swiss mice.**—Medium from mouse embryo cultures showing CPE after treatment with DMBA lymphosarcoma extract #1 was inoculated into newborn Swiss mice as described in “Methods.” Two control groups of animals were used. One received inoculations of medium from cultures treated with spleen extract #1, and the other was left untreated. The spleen cultures, as described in the previous section, showed only minor cell damage after exposure to the extract. The extract-treated groups of mice were inoculated with pooled media from the first, second, and third serial passages of the extracts.

Within 6–7 weeks mice from both spleen extract- and tumor extract-treated groups began to die, with symptoms of ruffled fur, loss of weight, and labored breathing. Deaths occurred more often in the tumor extract group than in the

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**TABLE 3**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>No. passages</th>
<th>No. cultures</th>
<th>No. showing CPE</th>
<th>Days to CPE</th>
<th>No. recovered*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen (1)</td>
<td>1–6</td>
<td>35</td>
<td>21*</td>
<td>3–14</td>
<td>35</td>
</tr>
<tr>
<td>Spleen (2)</td>
<td>1–6</td>
<td>19</td>
<td>0</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>Liver</td>
<td>1–6</td>
<td>20</td>
<td>0</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Kidney</td>
<td>1–6</td>
<td>20</td>
<td>2*</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>Thymus (1)</td>
<td>1–6</td>
<td>23</td>
<td>9*</td>
<td>3–5</td>
<td>23</td>
</tr>
<tr>
<td>Thymus (2)</td>
<td>1–6</td>
<td>18</td>
<td>0</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Lung (1)</td>
<td>1–3</td>
<td>12</td>
<td>11</td>
<td>3–5</td>
<td>1</td>
</tr>
<tr>
<td>Lung (2)</td>
<td>1–3</td>
<td>12</td>
<td>12</td>
<td>3–4</td>
<td>0</td>
</tr>
</tbody>
</table>

* Round, refractile cells appeared during early passages, but cultures recovered after medium changed.
spleen extract group. Grossly, the lungs showed plum-colored areas, sometimes involving the whole of one or more lobes. Microscopically the spleen and liver showed varying degrees of amyloidosis, and the lungs showed infiltration of the alveoli with plasma cells. Perivascular cuffing with plasma cells also occurred to a variable extent in the liver and kidney. As the infection progressed, large areas of concentration of these cells appeared in the lungs around blood vessels and bronchi (Fig. 4), and in advanced cases very little normal lung could be found, the whole lobe becoming filled with plasma-cell infiltration and focal necrosis. The opinions of three animal pathologists were that these were the lesions of endemic pneumonia. This disease is common in laboratory mice and rats but does not normally manifest itself until the animals are 40–50 weeks of age. The mortality is not usually high, and in our own colony about 10 per cent of the mice show gross or microscopic evidence of the disease at death. Endemic pneumonia and other chronic respiratory diseases of mice have been reviewed recently by Joshi et al. (8).

Table 4 shows the complete data of endemic pneumonia incidence in the three groups of mice. Mice dying of nonspecific causes during the first 30 days after birth are not included. Most of these deaths were due to cannibalism.

The incidence of endemic pneumonia in the tumor extract-treated group was significantly higher ($P < .01$ by the $\chi^2$ test) than in either the untreated or the spleen extract-treated groups, and cases with gross lung lesions appeared as early as 40 days. The incidence in the spleen extract-treated group was slightly higher than normal, and cases with gross lung lesions also appeared very early. It is felt that this was due to the presence of small amounts of virus in the spleen extract-treated cultures and to cross infection of one group of animals by another during handling. It was impossible to keep the different groups completely isolated from one another. However, since some cell damage did occur in early culture passages of the spleen extract used and since the untreated group appeared to follow the pattern of the rest of our mouse colony (which is kept in a separate building), it is probable that the presence of virus in the spleen extract was the major factor in the early cases seen in the spleen extract group.

The tumor incidences in the three groups are shown in Table 5. The number of animals in each group represents those surviving to 234 days (which was the age of the first mouse to die with a tumor). It can be seen that the incidences of tumor deaths in each group are very similar. When the tumor extract group is compared with either the spleen extract or control groups by the $\chi^2$ test the probabilities of the small differences being due to chance are between 0.8 and 0.5 for both comparisons.

Some animals developed more than one tumor, and if the total number of tumors appearing in the groups are compared the tumor extract appears to have raised the probability of tumor formation (third column, Table 5). Again, however, no significant differences appeared after statistical analysis by the $\chi^2$ test. The last two columns in Table 5 show the mean age of the animals dying with tumors and the range. The groups were almost identical in this respect. The lower part of Table 5 shows the distribution of the three different types of tumors found in these animals. Again, no differences were apparent between any of the groups.

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widespread. Cell-free extracts of this tumor were inoculated into twenty newborn hamsters, and after 1 year of observation no evidence of transmissibility is evident.

Properties of the virus isolated from DMBA-induced malignant lymphoma.—Several experiments were undertaken to determine some of the properties of the virus present in infected culture media after treatment with DMBA-induced lymphoma extracts. These included (a) heating in a water bath at 56°C for 1 hour and subsequent testing for capacity to produce CPE on mouse embryo cultures, (b) centrifugation in a Spinco preparative ultracentrifuge at 100,000 × g and subsequent testing for ability to produce CPE, (c) filtration through Millipore filters of 10-, 50-, and 100-mg pore size and testing of the filtrates for ability to produce CPE, (d) inoculation of cultures with serial tenfold dilutions of infected culture medium, (e) inoculation into the yolk sac of fertile eggs and testing of yolk sac after 6 days’ incubation for capacity to produce CPE, and (f) hemagglutination experiments with mouse, guinea pig, sheep, and chicken red cells.

A summary of these experiments is given in Table 7. The virus appeared to be highly active, of small size (between 10 and 50 μm) and resistant to heat inactivation. It failed to hemagglutinate any of the four types of cells used in the tests, and it produced typical endemic pneumonia lesions in both Swiss mice and Syrian hamsters.

**DISCUSSION**

The induction of malignant lymphomas and lung adenomas in newborn mice after injection of DMBA confirms the work of Pietra et al. (18), Stich (23), and Roe et al. (19). Although the foregoing experiments point to the localization of an endemic respiratory virus in the lymphoma tissue, no evidence of any oncogenic properties of this virus is evident.

**TABLE 7**

**Properties of Virus in the Medium of Mouse Embryo Cultures Showing Cytopathogenic Effect**

1. Resistant to heating to 56°C for 1 hour.
2. Only partially sedimented at 100,000 × g for 1 hour.
3. Particle size between 10 and 50 μm.
4. 10⁻⁷ dilution destroys mouse embryo cultures at 15th passage.
5. Survives two yolk-sac passages in fertile eggs.
6. Fails to hemagglutinate mouse, guinea pig, sheep, or chicken red cells.
7. Produces lung lesions in Swiss mice and Syrian hamsters diagnosed as endemic pneumonia.
virus was apparent. The use of serial tissue culture passages and newborn recipient animals should have given good opportunity for any tumor-inducing ability of the virus to become manifest. It is pertinent to note that Roe et al. (20) found no evidence of tumor-inducing activity in cell-free extracts of DMBA-induced lymphoma for strain 101 mice, and Miller (15) was unable to detect any oncogenic effects of similar extracts in DBA/2 mice. Taken together, these results seem to offer strong evidence against a specific tumor virus's being involved in the genesis of lymphoma resulting from exposure to DMBA. The demonstration of induction of tumor virus activity by x-radiation by Gross (7) and Lieberman and Kaplan (11) was a hopeful sign that a common denominator for cancer induction might indeed be viral, but the inclusion of chemically induced tumors in the scheme must await further evidence. The single lymphosarcoma arising in the hamster given an inoculation at birth of endemic pneumonia virus cannot be considered as definitely resulting from virus activity, even though the animal was only 4 months old when the tumor became apparent. Newborn hamsters given inoculations of cell-free extracts of this neoplasm have failed to develop tumors of any kind over a period of 1 year. It is well known, of course, that the continued presence of virus activity is not necessary for continued tumor growth in a number of virus-induced tumors including the Rous sarcoma and polyoma. It is possible that the presence of virus is transient and occurs early in the malignant process. Experiments to test this possibility are desirable.

Another possibility to consider is that of interaction between viral and chemical carcinogenic agents. A number of reports have appeared in the literature of a synergism between either oncolytic or oncogenic viruses and chemical carcinogens in the induction of tumors. M. Duran-Reynals (6) reported that the incidence of tumors induced by methylcholanthrene painting of mice was enhanced during vaccinia infection. Martin et al. (12) showed that infection with vaccinia, Coxsackie B., ECHO 9, or poliovirus all had an enhancing effect on the induction of tumors by one or another of various chemical carcinogens. Wisely (24) described similar effects in mice repeatedly exposed to respiratory viral infection. In previous years, F. Duran-Reynals (4) had shown that the painting of chickens and pigeons with methylcholanthrene resulted in the localization of fowl pox virus in lesions developing at the painted sites. The lesions sometimes became malignant, but only fowl pox virus could be isolated from them. He also showed (5) that in cortisone-treated mice given inoculations of vaccinia virus and previously painted with methylcholanthrene, tumors developed precisely at the site of inoculation.

Among the evidence for interaction between oncogenic viruses and chemical carcinogens can be cited the early reports of Rous and Kidd (21) and Kidd and Rous (9), who showed an enhancement in malignancy and number of tumors arising when Shope papilloma virus was injected into rabbits' ears previously painted with tar. Ahlstrom and Andrewes (1) also showed an increased malignancy in tumors induced by Shope fibroma virus if it was inoculated into rabbits treated with tar or other chemical carcinogens. More recently Rowson et al. (22) found that mice infected with polyoma virus when newborn and later treated with chemical carcinogens developed more polyoma tumors than did those receiving virus only. They also showed that skin tumors induced with 3,4-benzpyrene were greater in number, appeared earlier, and became more malignant if the mice had received polyoma virus at birth than if they had not. Polyoma virus did not, however, seem to exert any influence on the capacity of DMBA to produce tumors. This latter observation, perhaps, argues against polyoma virus's being involved in the genesis of the lymphomas resulting from DMBA in our experiments, although our Swiss mouse colony does carry polyoma antibody. Furthermore, other workers have been unable to find any evidence for a relationship between polyoma virus and lymphocytic tumors (8, 10).

That oncolytic virus infection can have an influence on chemical carcinogenesis seems, therefore, well documented, and it is possible that in these experiments an interaction similar to the

![Fig. 1.](image1) Monolayer of primary Swiss mouse embryo cells before treating with DMBA-induced lymphomas extract. X300.

![Fig. 2.](image2) Early cytopathogenic effect produced in mouse embryo monolayer by DMBA-induced lymphomas extract. Many cells at the periphery of the empty areas are granular and taking up a rounded form. X300.

![Fig. 3.](image3) A later stage in the destruction of the mouse embryo monolayer. Most cells are granular and rounded. Cultures that were affected to this extent did not recover. X300.

![Fig. 4.](image4) Section of the lung of a Swiss mouse with advanced endemic pneumonia resulting from inoculation with medium from cultures infected with DMBA-induced lymphomas extracts. Severe cuffing of the bronchi with plasma cells is apparent. Infiltration of the alveolar walls with similar cells is also obvious. X100.
type described by F. and M. Duran-Reynals between fowl pox or vaccinia and methylcholanthrene has occurred. In this kind of interaction a tumor eventually results, and the oncolytic virus can be isolated from the tumor. Experiments are under way in which combined endemic pneumonia virus and DMBA injections have been given to both cortisone-treated and untreated mice. The results will be presented in a later paper.

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