Variations in Polyoma Virus Genotype in Relation to Tumor Induction in Mice

Characterization of Wild Type Strains With Widely Differing Tumor Profiles

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The authors have explored the effects of variations in mouse polyoma virus genotype on patterns of tumor formation in the mouse. Four "wild type" virus strains were surveyed. Two were highly oncogenic, inducing multiple tumors of epithelial and mesenchymal origin, at high frequency and with short latency. The other two strains were weakly oncogenic, inducing fewer tumors, solely of mesenchymal origin, and after a long latency. These sharply contrasting tumor profiles were reproduced with virus stocks derived from molecularly cloned viral genomes. Though vastly different in their oncogenic properties, these cloned viruses proved equally effective in transforming established rat fibroblasts in culture and showed the same patterns of tumor antigen expression in cultured mouse cells. Complexes of polyoma middle T antigen and pp60^c-src were demonstrated in extracts of epithelial tumors induced by a highly oncogenic virus strain. It is concluded that polyoma viral genetic determinants for tumor induction in the mouse are more complex than those previously defined by the use of cell transformation systems. (Am J Pathol 1987, 127:243–261)

THE MOUSE polyoma virus was so named because of its remarkable ability to induce neoplasms in a wide variety of cell types in its natural host, Mus musculus.1 With some viral isolates, epithelial tumors are induced at high incidence in the major and minor salivary glands, the mammary glands, the hair follicles, and thymus.2–5 In addition, polyoma virus induces mesenchymal tumors of several cell types at a variety of anatomic sites.6–8 The large array of tumor types that may occur in response to mouse polyoma virus is so striking and clearly delineated that it has been referred to, in the aggregate, as the mouse polyoma tumor constellation7 (see Table 1, Part A).

Over 30 different cell types may be infected after inoculation of the newborn mouse with polyoma virus, and tumors may arise from more than a dozen of these. Table 1 summarizes observations pooled from several investigators on the distribution of lytic lesions and neoplasms in various tissues. A major site of virus replication and persistence is the kidney.9–11 In this organ, lytic lesions are found in both epithelial cells and mesenchyme, but only the latter undergoes neoplastic transformation and overt tumor development. Essentially all tissues in which neoplasms develop have also been found to show lytic lesions, but a variety of tissues and cell types, such as the exocrine
Table 1—Effects of Polyoma Virus on Various Cell Types in the Mouse

<table>
<thead>
<tr>
<th>A. Polyoma tumor constellation (the authors have also observed lytic viral effects in these cell types, with the exception of sweat gland epithelium)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epithelial</td>
</tr>
<tr>
<td>Salivary glands (major and accessory)</td>
</tr>
<tr>
<td>Thymus</td>
</tr>
<tr>
<td>Mammary glands</td>
</tr>
<tr>
<td>Hair follicle-sebaceous apparatus</td>
</tr>
<tr>
<td>Lacrimal glands</td>
</tr>
</tbody>
</table>

B. Lytic lesions, but no neoplasms (unpublished observations by authors, except as referenced)

Renal cortical tubules, urothelium3-5,9 (lesions sometimes classified as preneoplastic)
Pancreas (exocrine)
Lung (bronchial epithelium)4 (primary lung tumors reported4-48)
Liver parenchyma and ductal epithelium (sporadic)
Myometrium (sarcomas reported in athymic nude mice32-37)
Vascular smooth muscle (aorta and large arteries)
Cardiac muscle
Skeletal muscle
Cartilage
Substantia propria, gut
Sertoli cells, testis
Gland (specific types indeterminate)
Kupffer cells (?)
Histiocytes/macrophages

C. No lytic or neoplastic lesions reported as yet in literature or observed by authors

Mature neurons and ganglion cells
Retina and other eye tissues, including lens and cornea
Meninges
Jacobson’s organ
Ovary
Fallopian tube epithelium
Endometrial epithelium and stroma
Cervix, vagina, vulval glands
Preputial glands
Esophagus, stomach and intestinal epithelium, Brunner’s glands
Pancreatic islets
Leydig cells, testis
Adrenal cortex
Parathyroid glands
Bladder (anterior and posterior)
Pineal gland
Epidermis
Oropharynx mucosa
Gall bladder
Middle ear epithelium
Cerumin glands
Hemic cells (erythrolytic, granulocytic, and megakaryocytic series)

*After subcutaneous or intraperitoneal inoculation of newborn mice.

pancreas, testis, and urothelium, show only lytic lesions. The cellular and developmental biologic basis for these selective interactions with the virus are not understood, although the phenomenon has long been a subject of investigation.8,9,12-15

Using virus grown to high titer in mouse cell cultures, transformation of mouse, hamster, and rat cells can be accomplished in vitro. Organotypic cultures of mouse salivary glands have been used,16 as well as dissociated cell cultures of mouse, hamster, or rat embryos, in which fibroblastic cells predominate.6,17-19 Established rodent fibroblast cell lines have also been used as targets for in vitro transformation.20-22 The hamster and rat cell systems have been favored in studies of transformation, chiefly because these cells are nonpermissive and therefore free from complications due to continued lytic infection as occurs in the mouse cell and organotypic cultures.23 Such in vitro cell transformation systems are less time-consuming and less costly than tumor induction and are ostensibly a more direct measure of the transforming activity of the virus.

The availability of cell culture systems for virus propagation and cell transformation has made possible detailed molecular biologic and genetic studies of the virus.24 An important conclusion emerging from these studies is that the formation of a complex between the polyoma virus-encoded middle T antigen and the cell-encoded proto-oncogene product pp60c-src at the plasma membrane is critical for cell transformation.25 This interaction, leading to activation of the tyrosine-specific protein kinase activity of pp60c-src26 is presumed to be important in tumor induction, although the presence of these complexes in tumors has not been reported. Implicit in the use of in vitro transformation systems is the assumption that, at the target cell level, the molecular interactions between cell and virus are the same as, or at least very similar to, those in vivo, regardless of species and tissue origin of the target cell. This assumption has not been seriously challenged experimentally.

Over the years, many independent isolations of mouse polyoma virus were made.5,23,27-31 These wild type strains were not always assayed for tumor-inducing activity in mice, and in those cases where they were, different inbred strains of mice were usually used by different investigators.6 Many studies32-34 made it clear that differences in the tumor response among different mouse strains could be attributed for the most part to differences in the cellular immune responses of the strains tested. Generally, from one investigation to another, not only the strain of mouse but also the strain and dose of the virus were varied or remained indeterminate, making it impossible to
ascertain whether or not differences in tumor tropisms could be attributed to differences between virus strains.

In the course of comparing tumor induction by two independently isolated virus strains in a single strain of inbred mouse, we were struck by the observation that uncloned stocks of these two viruses had greatly disparate potentials with respect to overall tumor incidence, the type of tumor they could induce, and the latent period required for tumors to appear and cause mortality. On the one hand, strain PTA, isolated originally from a retrovirus-induced leukemia and used extensively in studies with salivary gland organotypic cultures, was seen to induce a high incidence and wide variety of both epithelial and mesenchymal tumors within a short latent period after subcutaneous inoculation into newborns. On the other hand, strain RA, a tissue culture adapted strain serving as a wild type virus control in genetic studies of transformation, when similarly inoculated into mice of the same strain and age, and at equal dosage, induced only two types of mesenchymal tumors in a low percentage of the recipients, after long latent periods. This result could not have been predicted on the basis of the transforming properties of the two virus strains in vitro, nor by assays of the viral middle T antigen-associated protein kinase activity, both of which proved to be the same for the two viruses.

It is reasonable to postulate genetic difference between the two virus populations to account for the vastly different patterns of tumor induction in hosts of uniform genetic constitution. Genetic heterogeneity within a single virus stock is known to exist, and could conceivably contribute to heterogeneity in the tumor profile, particularly for PTA, which has such broad biologic potential. Diversity in virus genotype could account for differences in pathogenetic processes at many levels, including 1) direct interaction of the virus with target cells, either at the cell surface (receptor recognition) or intracellularly, 2) virus replication rates and levels of viremia established prior to target cell transformation, 3) immunologic properties of the virus resulting in differences in neutralization, and 4) immunologic rejection of transformed cells depending on the potency of the virus' tumor-specific transplantation antigen.

To investigate the role of polyoma virus genotype in tumor induction as distinct from cell transformation and to begin to explore possible mechanisms would require the following initial steps: 1) isolation and propagation of homogeneous populations of virus from each of the parental strains by molecular cloning, 2) comparative pathologic studies in a single strain of inbred mouse to establish whether or not the cloned viruses induced different tumor profiles, and 3) comparative studies of the two cloned virus strains in vitro including virus growth and cell transformation and expression of the known viral tumor antigens.

In this paper we report results of these initial steps that clearly demonstrate effects of mouse polyoma virus genotype on patterns of tumor induction in the mouse. In particular, the results suggest that viral genetic determinants additional to those recognized as critical in cell transformation come into play in the animal. Directions are outlined for clarifying how variations in virus genotype might produce variable effects on pathogenetic processes leading to different tumor profiles.

Materials and Methods

Mice and Virus Injections

All mice were of inbred strain C3H/BiDa, from our cesarean-derived, polyoma virus-free breeding colony. Litters of newborns were inoculated at least as early as 18 hours of age by injection of 0.05 ml of virus suspension subcutaneously in the dorsal midline, using a 1.0-ml tuberculin syringe and 27-gauge hypodermic needle. Only those recipients that survived beyond 1 month of age were included in scorings for tumor development. Unless otherwise noted in the tables, the dose of virus was 5 \times 10^6 to 2 \times 10^7 plaque-forming units (pfu) per animal.

Pathology

Development of tumors was detected by twice-weekly gross inspection of recipients. Overdose of diethyl ether anesthesia via inhalation was used to kill the mice. In order to allow them to develop as many tumor types as possible, we delayed sacrifice of tumor-bearing animals until it was apparent that they would survive only for a matter of hours or a few days longer. Mice without tumor were killed at approximately 1 year. Of the 242 mice represented in the tables, 14 were found dead, but were necropsied as soon as possible. In a few of these cases, the dead were partially eaten by cagemates, causing loss of some tumors that could therefore not be verified microscopically.

Tumors and tissues were fixed in Bouin’s fixative for 18–24 hours, after which they were placed in 70% ethanol until dehydration and embedding in Paraplast. Sections were cut at 5-mm thickness and stained with Harris’s hematoxylin and eosin. Sections were examined from all grossly detected tumors, and
from the following tissues and organs, regardless of whether they contained grossly visible tumors: heart, lungs, thymus, all three pairs of major salivary glands, extraorbital lacrimal glands, thyroid gland, liver, pancreas, spleen, stomach and duodenum, both kidneys, both adrenal glands; ovaries, uterus, and cervix in females; testes, epididymi, urethra, prostate, bulbourethral glands, and coagulating glands in males; and urinary bladder. Sections often included mediastinal, renal, and cervical lymph nodes. In the case of small organs such as thyroid gland, adrenal glands, and glands of the male genitourinary tract, these organs were sometimes missed by the microtome knife and were not invariably available at microscopy.

Virus Strains

Uncloned Viruses

The PTA strain was derived, as diagrammed previously, from a leukemia induced in a C3H/Gr mouse by Dr. L. Gross with an extract from an AKR "spontaneous" mouse leukemia. From 1957 to 1982 the PTA strain was propagated only in the continuous mouse macrophage tumor cell line P388D1, as described. This virus strain has sometimes been referred to as the "P388D1 strain." RA, originally designated "NG59-RA," was reconstructed to wild type from the hr-t mutant NG59 by restriction fragment marker rescue and originated from the Pasadena small plaque strain. The strains A2 and A3 have been extensively used, and the nucleotide sequences of their genomes have been determined.

Cloning of Viruses

The strains PTA and RA were molecularly cloned into Escherichia coli. Viral DNAs were isolated by the method of Hirt and purified by equilibrium centrifugation in cesium chloride density gradients containing 200 μg/ml ethidium bromide. The viral DNAs were digested with BamHI (New England Biolabs), ligated to BamHI-cut pBR322, and used to transfect CaCl2-treated HB101. The cloned viral DNAs were amplified in E coli as described. The viral DNAs were excised from the vector DNA by BamHI digestion, ligated under dilute conditions for promotion of self-closure, and used to transfect primary baby mouse kidney cells (BMKs) by the calcium phosphate procedure. The reconstructed viruses were plaque-purified on NIH-3T3 cells. Virus stocks were grown on BMK cells from polyoma virus-free suckling CD-1 mice, titered by plaque assay on NIH-3T3 cells, and used directly for inoculation as described above.

Assays of Virus Growth and Cell Transformation

Virus growth was assayed as described. Briefly, 10⁵ NIH-3T3 or UC1-B cells were seeded on 35-mm diameter plastic Petri dishes; the following day, the cells were infected with virus at a multiplicity of infection of about 0.1 pfu/cell. At 90 hours after infection, the cultures were harvested by freeze thawing and titered by plaque assays with UC1-B as the indicator cells. The burst size is taken as the ratio of virus output at 90 hours divided by the virus input.

Transformation assays were done as previously described. Thirty-five-millimeter dishes containing 2 × 10⁵ F-111 rat fibroblast cells were infected with virus of known titer. Approximately 20 hours after infection, the cells were removed from the plates with trypsin, two-thirds of the cells were transferred to 60-mm diameter plates for development of foci, and one-third were suspended in soft agar. Ten to 14 days later, macroscopic foci and soft agar colonies were counted.

Analyses of Polyoma Tumor Antigens

The procedures for labeling cells, immunoprecipitation and analysis of T antigens have been previously described. Briefly, NIH-3T3 cells were labeled with [35S]-methionine (Amersham) at 100 μCi/ml in Hanks' balanced salt solution for 2 hours. The T antigens were extracted for 20 minutes at 4°C in 1 ml of lysis buffer (0.137 M NaCl, 0.02 M Tris-HCl pH 9.0, 0.001 M MgCl₂, 0.001 M CaCl₂, 10% glycerol and 1% [vol/vol] Nonidet P-40). The extracts were cleared by centrifugation, and antibody to the polyoma tumor antigens in the form of anti-T ascites was added to the extract. S aureus protein A–sepharose (Pharmacia) was added and the mixture gently agitated for 1 hour. The precipitates were washed first with cold phosphate-buffered saline, next with 0.5 M LiCl–0.1 M Tris-HCl (pH 6.8), and finally with distilled water.

In vitro kinase reactions were performed with the use of washed immunoprecipitates as described. Tumors used for the in vitro kinase reactions were stored frozen at −70°C. Tumors or normal tissues were minced and homogenized in lysis buffer containing 100 μg/ml aprotinin, 100 μg/ml leupeptin, and 0.2 mM phenyl-methanesulfonylfluoride. Protein concentrations in the extracts were determined, and immunoprecipitations were performed on 200 μg protein. The antiserum used was either anti-T ascites or anti-pp60c-src monoclonal 327.51 The immunoprecipitates were collected and washed as described above. Kinase reactions were performed in 0.15 ml of kinase buffer (0.02 M Tris-HCl, pH 7.5, 0.005 MgCl₂)
with 20 μCi γ-32P ATP (ICN) for 15 minutes at room temperature. The precipitates were then washed again as previously described.49

The washed immunoprecipitates were boiled in sample buffer (2% SDS, 5% β-mercaptoethanol, 0.0625 M Tris-HCl pH 7.8, 10% glycerol) and analyzed by SDS polyacrylamide gel electrophoresis.52 Gels were fixed (50% methanol, 10% acetic acid), dried, and analyzed by autoradiography.53 Alkali treatment of gels was performed by incubating the fixed gel in 1 M NaOH at 65 C for 1.5 hours.54 The treated gel was washed several times in H2O, refixed, dried and exposed to X-Ray film.

**Results**

Pathologic Findings on Tumor-Inducing Activity of Different Virus Strains

For convenience, we refer to tumors found at the gross level of inspection as overt tumors and to those found only at the microscopic level as occult tumors. Only the four most common sites of epithelial tumors and the four most common sites of mesenchymal tumors are tabulated. For virus strains PTA and A2, overt tumors were occasionally found also in the adrenal medulla, thyroid gland, and dental organ (ameloblastoma), and occult tumors were detected in periurethral glands, bulbourethral glands, and prostate gland as well.

Different Polyoma Virus Isolates Show Different Patterns of Tumor Induction

Tumor profiles of four virus isolates were established. Table 2 presents the data based on gross detection only, since this level of resolution more fully satisfies the question as to whether fully developed and actively growing neoplasms were present.

Three relationships are apparent in Table 2. First, strains PTA and A2 appear similar to each other, in that both strains induced high frequencies of epithelial tumors as well as lower frequencies of mesenchymal tumors. On the other hand, RA and A3 appear similar to each other, but different from PTA and A2, in that they induced no epithelial tumors and induced mesenchymal tumors at relatively low frequencies. The possibility that occult epithelial tumors may have been present in mice inoculated with either of the weakly oncogenic strains RA and A3 is remote in view of our failure to find any occult epithelial tumors in a sample of more than two-thirds of the mice constituting these two groups. No renal medullary tumors and only a single bone tumor were recorded in mice inoculated with RA or A3, whereas these tumors occurred with substantial frequency in PTA- and A2-inoculated animals. Aside from mammary tumors, which are roughly three times more frequent in females, compared with males, there is no sex predilection in tumor frequency elsewhere in the tumor profile.

Second, the highly oncogenic strains PTA and A2 had one outstanding difference between them, namely, that no overt thymic epitheliomas were induced by A3, whereas this tumor type was induced in 29 of the 32 mice inoculated with PTA. This difference is not an absolute one, however, because microscopic examination of the thymus revealed occult thymic epitheliomas in two of the 23 mice inoculated with the A2 strain. Even if occult tumors are accorded the same significance as overt tumors, a large and statistically significant difference is still evident between PTA and A2 with respect to induction of thymic epithelioma (91% versus 9%). This difference is unique and unrelated to induction of other epithelial tumors, because the rank order and frequencies for the other three major epithelial tumor types are similar for the two virus strains.

Third, Table 2 records a distinction between strains PTA and A2 on the one hand and strains RA and A3

<table>
<thead>
<tr>
<th>Tumor Profile</th>
<th>PTA</th>
<th>RA</th>
<th>A2</th>
<th>A3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ear Follicle</td>
<td>32/32</td>
<td>12/30</td>
<td>23/23</td>
<td>7/22</td>
</tr>
<tr>
<td>Thymus</td>
<td>82</td>
<td>304</td>
<td>113</td>
<td>305</td>
</tr>
<tr>
<td>Mammary Gland</td>
<td>42-125</td>
<td>266-476</td>
<td>72-314</td>
<td>231-370</td>
</tr>
<tr>
<td>Salivary Gland</td>
<td>23 (72)</td>
<td>18 (78)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fibrosarcoma</td>
<td>1 (3)</td>
<td>8 (27)</td>
<td>1 (4)</td>
<td>7 (32)</td>
</tr>
<tr>
<td>Hemangioma</td>
<td>1 (3)</td>
<td>4 (13)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Renal Medulla</td>
<td>7 (27)</td>
<td>3 (13)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bone</td>
<td>6 (19)</td>
<td>0</td>
<td>12 (52)</td>
<td>1 (4)</td>
</tr>
</tbody>
</table>

*Numbers in parentheses indicate the percentage of animals in each group with a given tumor.
on the other, in that the survival times after inoculation were shorter for mice that received PTA or A₂ than for mice that received RA or A₃. In the case of strain PTA, this difference can be accounted for by the early appearance and rapidly lethal effects of the thymic epitheliomas that occurred in 29 of the 32 mice. However, no overt thymic tumors were found in A₂-inoculated mice. Although the mean survival time of A₂-inoculated mice was 31 days longer than that of PTA-inoculated mice, it was still approximately 190 days shorter than the survival time of mice that received either the RA or A₂ virus strain. This was due in large part to the late appearance, slow growth, and noninvolvement of vital organs by the subcutaneous sarcomas, the most common tumor type in RA- and A₂-inoculated mice. For the most part, tumors of salivary glands, hair follicles, and mammary glands were noninvasive, even though they grew rather rapidly and attained large size. Only rarely were pulmonary metastases observed from salivary tumors (2 cases), mammary tumors (1 case), and adrenal medullary tumors (1 case). No metastatic spread from hair follicle tumors was seen. Subcutaneous sarcoma sometimes reached a weight greater than that of the host before causing moribundity. In contrast, very small hemangiomas caused death at 119 days and 120 days in 2 mice. In 1 mouse, the trachea was obstructed by compression caused by hemorrhage from a hemangioma located adjacent to the thyroid gland. In the other, hemorrhage occurred from an intracerebral hemangioma.

In mice that received PTA, epithelial tumors were frequently multiple within a single organ and frequently occurred in multiple organs within a single mouse. In 11 of 32 mice, overt epithelial tumors were present at necropsy in all four of the most common sites (hair follicles, thymus, salivary glands, and mammary glands). In 25 of 32 mice, overt epithelial tumors were present at necropsy in at least three of the above sites; and in all 32 mice they were present at necropsy in at least two of the above sites (data not shown).

It should be noted that among the mice that approached or exceeded 1 year of age in the group inoculated with RA, 5 were found at necropsy to have well-differentiated hepatocellular neoplasms. These had no relation to the viral infection, because they occur at approximately the same frequency in old breeders, particularly the males, in our polyoma virus-free colony of C₃H/BiDa mice. Also, 2 female mice in the RA group were found to have mammary tumors of nonpolyoma type at necropsy. One was an adenoacanthoma, the other a ductal adenocarcinoma of a type associated with hyperplastic alveolar nodules. Both were histologically distinguishable from polyoma type mammary tumors, and both types are known to occur in old female mice in our colony.

**Essential Features of the Tumor Induction Profiles of PTA and RA Are Stable Upon Molecular Cloning of the Viral Genomes**

The strongly oncogenic strain PTA and the weakly oncogenic strain RA were molecularly cloned as described in Materials and Methods, plaque-purified, grown to high titer, and inoculated into newborn mice. The results in Table 3 show a large degree of congruity between the tumor profiles induced by the cloned viruses and their uncloned progenitors (Table 2). The cloned PTA virus induced epithelial tumors in the same set of organs as did the uncloned PTA, and cloned RA virus, like its uncloned parent strain, failed to induce any epithelial tumors.

Minor differences were found, however, between cloned and uncloned virus preparations. With the cloned RA virus, subcutaneous sarcomas were reduced by about half (4 of 33, compared with 8 of 30), and hemangiomas were absent where there had been 4 among 30 mice inoculated with uncloned RA. For the cloned PTA group, there were reductions in relative tumor frequencies for tumors of thymus, salivary glands, mammary glands, and hair follicles. At the same time, bone tumors and subcutaneous sarcomas were increased. All of these differences correlate with a longer mean survival time for the cloned PTA versus the uncloned PTA recipients (117 days versus 82 days). Probably because the mice that received cloned

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Table 3 — Tumor Induction Profiles of Cloned PTA and RA Viruses

<table>
<thead>
<tr>
<th></th>
<th>RA cloned</th>
<th>PTA cloned</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction of mice with tumor(s)</td>
<td>4/33</td>
<td>37/37</td>
</tr>
<tr>
<td>Mean age at necropsy</td>
<td>429</td>
<td>117</td>
</tr>
<tr>
<td>Age range at necropsy</td>
<td>323–530</td>
<td>45–225</td>
</tr>
<tr>
<td>Epithelial tumors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hair follicle</td>
<td>0</td>
<td>33 (89)†</td>
</tr>
<tr>
<td>Thymus</td>
<td>0</td>
<td>30 (81)</td>
</tr>
<tr>
<td>Mammary gland</td>
<td>0</td>
<td>9 (25)</td>
</tr>
<tr>
<td>Salivary gland</td>
<td>0</td>
<td>6 (16)</td>
</tr>
<tr>
<td>Mesenchymal tumors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrosarcoma</td>
<td>4 (12)</td>
<td>7 (19)</td>
</tr>
<tr>
<td>Hemangioma</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Renal medulla</td>
<td>0</td>
<td>4 (11)</td>
</tr>
<tr>
<td>Bone</td>
<td>0</td>
<td>10 (28)</td>
</tr>
</tbody>
</table>

*Because complete histologic examination of all mice inoculated with the uncloned RA virus (Table 2) revealed no additional tumors, histologic examination of grossly negative organs were limited initially to one-third of the mice in this group. No tumors were found in this histologically examined sample either; so the remaining mice in this group were examined only grossly at necropsy, as a cost-saving measure.

†Numbers in parentheses indicate the percentage of animals in each group with a given tumor.
virus developed fewer epithelial tumors, they survived longer and thus had sufficient time to develop more of the late-appearing subcutaneous sarcomas and bone tumors. Considering only those mice with overt thymic epitheliomas, which impose the most serious and acute threat to life, those that received uncloned PTA survived a mean of 75 days, whereas those that received cloned PTA survived a mean of 109 days. In both instances, these means are lower than the means for each of the entire respective groups (82 days and 117 days). Mean survival times for mice without overt thymic epitheliomas were 101 days for the uncloned PTA recipients and 232 days for the cloned PTA recipients, in both cases higher than the means for mice with overt thymic epitheliomas in the corresponding virus-recipient groups, and also higher than the means for the corresponding entire virus-recipient groups. Therefore, for the PTA-infected mice, there is an inverse relationship between the frequency of overt thymic epitheliomas and mean survival time, as well as between frequency of overt thymic epitheliomas and frequency of overt subcutaneous sarcomas and bone tumors.

On the contrary, a direct, rather than an inverse, relationship is seen between frequency of thymic epitheliomas and frequency of epithelial tumors of salivary glands, mammary glands, and hair follicles. Incidences were reduced for tumors at all of the major epithelial sites in the group that received cloned PTA, as compared with the group that received uncloned PTA. The reduction of incidence was not equal in degree for all of the epithelial sites, however. For thymic and hair follicle tumors the reduction was only 10% and 11%, respectively, whereas for mammary gland and salivary gland tumors it was 25% and 56%, respectively.

The incidence of overt renal medullary tumors was not significantly reduced in the group that received cloned PTA. The mean survival time at necropsy for mice with overt renal medullary tumors was 95 days for those that received uncloned PTA and 120 days for those that received cloned PTA. The earliest age at which an overt tumor of this type was seen in either group was 82 days, and the oldest, 136 days. Thus, this tumor type appears to be temporally positioned between the early-appearing epithelial tumors and the late-appearing subcutaneous sarcomas.

Lower Doses of the PTA Virus Still Induce a High Tumor Profile

The marked differences between RA and PTA might result simply from differing viral replication rates following inoculation of comparable doses into newborn mice. A virus with a low replication rate might be expected to cause a less severe and more slowly developing viremia. Consequently, fewer virus particles would reach the target sites than in the case of a virus with a high replication rate, and fewer transforming events would ensue. If this were true, it should be possible to reproduce the RA-type profile by using lower doses of PTA.

To examine this possibility, we inoculated groups of newborn mice with a series of 10-fold dilutions of the cloned PTA virus. The results in Table 4 show that reduction of dose by 1000-fold resulted in little alteration in the tumor profile. Although the numbers of mice used in this experiment were smaller than in our standard bioassay, the results were sufficiently clear to indicate that large reductions of the inoculated dose of PTA did not result in substantial change of the PTA tumor profile. The absence of mammary tumors in Column 2 is due to a fortuitous combination of factors, including small sample size with a predominance of males (4 of 6) and the fact that the 2 females

<table>
<thead>
<tr>
<th>Table 4 — Effect of Dosage of PTA Virus on Tumor Induction</th>
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</thead>
<tbody>
<tr>
<td>5 × 10⁵ pfu PTA</td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>Fraction of mice with tumor(s)</td>
</tr>
<tr>
<td>Mean age at necropsy (days)</td>
</tr>
<tr>
<td>Age range at necropsy (days)</td>
</tr>
<tr>
<td>Epithelial tumors</td>
</tr>
<tr>
<td>Hair follicle</td>
</tr>
<tr>
<td>Thymus</td>
</tr>
<tr>
<td>Mammary gland</td>
</tr>
<tr>
<td>Salivary gland</td>
</tr>
<tr>
<td>Mesenchymal tumors</td>
</tr>
<tr>
<td>Fibrosarcoma (subcutaneous)</td>
</tr>
<tr>
<td>Hemangioma, any site</td>
</tr>
<tr>
<td>Bone</td>
</tr>
<tr>
<td>Renal medulla</td>
</tr>
</tbody>
</table>

*Numbers in parentheses indicate the percentage of animals in each group with a given tumor.
were sacrificed early because of thymic tumors. That there is no loss of mammary tropism on virus dilution is clearly evident from the results in Columns 3 and 4.

The increase in survival times and the reduction in frequencies of epithelial tumors in mice that received the lower doses of PTA indicate some dose-response relationship, though only minor in degree, within the range tested. It is possible that still greater dose reductions of PTA might lead to tumor responses similar to those seen with high doses of RA; this may be surmised from the fact that the 3 mice inoculated with cloned PTA (Table 3) which had the longest survival periods (279, 280, and 344 days) bore only subcutaneous sarcomas, thus resembling mice inoculated with high doses of RA. We speculate that these 3 mice received an exceptionally low effective dose of PTA as a result of escape of most of the inoculum after withdrawal of the hypodermic needle, an inadvertent event that was occasionally seen to occur. Further investigation is needed to address the role of virus replication rates in vivo on the tumor profiles and how PTA and RA may differ in their replication in the animal.

Histologic Findings on Overt and Occult Tumors

All of the tumor types found in overt phase in this study have been previously reported and adequately described in the earlier literature on polyoma virus tumors in mice.\(^5\)\(^-\)\(^5\)\(^8\)\(^-\)\(^5\)\(^9\) \(^5\)\(^0\) Example of each of the four major epithelial types listed in the tables are illustrated in Figures 1–8, and of the four mesenchymal types, in Figures 11–14. Observations to follow are limited to some that have not been recorded previously or appear to have particular relevance to polyoma tumor pathogenesis and to differences between the tumor profiles induced by different viral strains.

Tumors in the occult phase were numerous in mice inoculated with the highly oncogenic PTA and \(A_2\) that induced epithelial as well as mesenchymal tumors, but were not seen in mice inoculated with the weakly oncogenic RA and \(A_3\) viruses that induced mesenchymal tumors only. The inclusion of occult tumors with overt tumors therefore alters the numeric data upward for virus strains PTA and \(A_2\), but not for strains RA and \(A_3\). Table 5 demonstrates this, using cloned and uncloned PTA as an example. (The data in Table 5 are based on the same series of animals as described in Tables 2 and 3.) In mice that received uncloned virus, 33 occult tumors of the various epithelial types were found by microscopic examination of organs that bore no overt tumors. In mice that received cloned PTA, 24 occult tumors were found at microscopy. The overall pattern of rare and occult tumors remained the same after cloning of the virus. Many microscopic lesions histologically identical with occult tumors were found coexistent with one or more overt tumors in a given organ in an individual mouse. These are not included in Table 5, because their quantitation is difficult and unreliable without complete serial sectioning. Their presence, however, is convincing evidence of the frequent multicentricity of polyoma tumors. They also provide evidence that in a given organ, tumors may be found in many different morphologic stages of development.

Although mentioned in the literature,\(^4\) it has not been widely recognized that occult epithelial tumors can be induced by polyoma virus in the male urogenital tract, specifically in the perirethral glands,\(^4\) bulbourethral glands (Figure 9), and prostate gland (Figure 10). We have not encountered a grossly detectable tumor at any of these sites, however, giving rise to uncertainty as to the pathobiologic potential of these lesions. Invariably, mice with occult tumors at these sites also bore other epithelial overt tumors responsible for their moribund condition.

Occult epithelial tumors could be classified in two categories or types of lesions, which were distinguished as follows: 1) Lesions detectable only with the aid of the microscope, but histologically identical to overt, actively growing tumors, appeared to be fully developed neoplasms,\(^5\)\(^9\) which were simply small at the time of necropsy. As a group, they tended to be larger than occult lesions of Category 2 below, and were composed of nearly pure populations of neo-

---

Figure 1—An overt tumor of the parotid salivary gland. Like this example, most polyoma virus-induced salivary gland tumors are pleomorphic, being composed of a mixture of distinctly epithelial cells forming gland- and ductule structures, together with elongated mesenchymelike cells weaving among the epithelial components. The mesenchymoid component has been shown to be part of the neoplastic cell population, and not stromal in nature.\(^1\) These tumors thus resemble human salivary gland tumors classified as "mixed tumors" or "pleomorphic adenomas." Unlike some human mixed tumors, however, the polyoma-induced tumors in mice do not contain squamous epithelial components or cartilage-like foci. Rarely, they have metastasized to lung. (H & E, X100)

Figure 2—Example of an occult salivary gland tumor of Category 1 (see text). The tumor is composed of epithelial and mesenchymelike components and is similar to the overt tumor shown in Figure 1 except for size. Small aggregates of lymphocytes rim the periphery of the tumor (arrow) and may represent cellular immune response to tumor antigens. Normal sublingual salivary gland is on the right. (H & E, X70)

Figure 3—Example of an occult salivary gland lesion of Category 2 (see text). The focus of altered epithelium is heavily infiltrated and surrounded by lymphocytes and plasma cells indicative of response to viral and/or tumor-associated antigens, as well as to nonspecific products of cell necrosis. Normal submandibular salivary gland surrounds the lesion. (H & E, X100)

Figure 4—Polyoma virus-induced thymic epitheloma. In lower right part of photo, the tumor cells form distinctly epithelial columns and clusters, whereas in upper left, the tumor displays little structure but contains diffusely distributed thymic lymphocytes entrapped within tumor. This tumor type has not been observed to metastasize. It sometimes occurs in ectopic cervical thymus. (H & E, X100)

Figure 5—Polyoma virus-induced thymic epitheloma at a sharp interface between tumor cells (left) and normal thymic cortex composed mainly of normal thymic lymphocytes (right). Tumor shows little structuring and is traversed here by a large capillary. Neoplastic epithelial cells have relatively abundant cytoplasm and are frequently seen in mitosis (arrows). (H & E, X250)
plastic cells (Figure 2). Only rarely did one see in these lesions evidence of viral lytic effects in the form of nuclear ballooning, margination of nuclear chromatin, intranuclear inclusion bodies, or karyorrhexis. Mitoses were approximately as numerous as in overt neoplasms, and evidence of cellular immune response in the form of peripheral aggregates of lymphocytes and plasma cells was slight or absent. 2) Lesions generally smaller in size than those in Category 1, and composed of mixtures of lytically infected cells and apparently fully transformed, proliferating cells. Features of lytically infected cells were as cited above for Category 1, and were seen in abundance. Mitoses were present, but less frequent, and peripheral aggregates of lymphocytes and/or plasma cells were often prominent. The fate of lesions in this category is ambiguous, as one is at a loss to know whether viral lytic processes, together with the cellular immune response, or the proliferation of transformed cells would have dominated in the two opposing processes, had the host survived longer. We refer to these lesions (Figure 3) as transitional/ambivalent, on the assumption that some have the potential to develop into neoplasms while others do not.

The PTA Tumor Profile Emerges in Mice Doubly Inoculated With PTA and RA Viruses

It is possible that the low tumor profile of RA is due to some aspect of its interaction with the host that abrogates tumor induction, rather than to an intrinsically low transforming potential of this virus in vivo. For example, RA may be more effective than PTA in inducing interferon, or in its interactions with the host immune system, either at the humoral level by rapidly inducing neutralizing antibody, or at the cellular level by inducing a strong anti-TSTA response.

To investigate the possibility of a protective effect of RA that would operate systemically to lower the tumor incidence, newborn mice were inoculated with a mixture of equal titers of PTA and RA viruses together. As shown in Table 6, the high tumor profile typical of PTA is seen, similar to that obtained with PTA alone (see Tables 2–4). These results thus provide no evidence for a protective effect induced by RA.

Comparisons of Cloned PTA and RA Virus Strains in Cell Cultures

PTA and RA Are Indistinguishable in Virus Growth and Cell Transformation Properties In Vitro

Virus growth was tested in NIH-3T3 and UC1-B, two established mouse embryo fibroblast cell lines commonly used for productive infection by polyoma virus. Infected cultures were harvested at 90 hours after infection, and the total virus yields were measured by plaque assay. The single cycle burst sizes for two experiments are shown in Table 7, Part A. In both cell lines, the two viruses replicate to comparable levels. Comparisons of burst size on UC1-B versus NIH-3T3 provide a test for possible defective function in the viral hr-t gene encoding the small and middle T (tumor) antigens; UC1-B cells are known to be "permissive" for hr-t mutants; ie, they partially bypass hr-t gene defects in the virus, while NIH-3T3 cells are "nonpermissive." The results showing equivalent burst sizes in these two cell lines are an indication that both PTA and RA have normal hr-t gene function for virus growth.

Tests for cell transformation by PTA and RA were carried out using an established rat embryo fibroblast cell line. Transformation was measured both by focus formation on monolayers and by growth in soft agar. Results of two experiments are shown in Part B of Table 7. The two viruses transformed with equal efficiencies in both assays, as shown by the comparable tfu/pfu (transforming units/plaque forming units) ratios. In ten such experiments, the average of the tfu/pfu ratios was 2.8 × 10^-4 for PTA and 1.8 × 10^-4 for RA.

Two viral genes are essential for cell transformation by polyoma virus: the ts-a gene encoding the large T antigen which functions primarily to initiate transformation, probably by facilitating integration of the viral DNA, and the hr-t gene encoding the middle and

Figure 6-A small, early polyoma virus-induced tumor originating from hair follicle epithelium. The proliferating epithelium forms a bulbous enlargement of the deeper half of the follicle and entraps nests of normal sebaceous gland cells (arrow). Epidermis and the more superficial segment of the follicle are not involved. Striated muscle fibers of the panniculus carnosus to lower left. (H & E, ×100)

Figure 7-The deep portion of a larger tumor of hair follicle origin. These tumors have an organoid structure, displaying persistence of the ability of the epithelium to form folliculike units (lower right) which produce soft keratinaceous material, rather than compact, cylindrical hair shafts. At the upper left the tumor expands into a keratin-filled sac lined by viable, proliferating epithelium. Arrows indicate capillary blood supply. These tumors are not invasive and have not been observed to metastasize. Central keratinaceous portions sometimes calcify, and the tumors have been likened to the benign calcifying epitheliomas of Malherbe found in humans. However, the polyoma-induced tumors often contain intranuclear Type A inclusion bodies. (H & E, ×70)

Figure 8—Portion of a polyoma-induced mammary tumor (lower part of field). Small, irregular glandlike structures are shown, as well as less structured cell masses. These tumors arise within mammary ducts, are often multicentric, and tend to fill and expand much of the duct system of an entire gland before breaking out of ducts. They have occasionally metastasized to lung. In the upper part of field are ducts within an adjacent lobe of mammary gland, some of whose cells contain ballooning nuclei (arrow) indicative of lytic virus infection. (H & E, ×250)

Figure 9—Portion of an occult tumor in a bulbourethral gland of a male mouse. Only occult tumors have been found at this site. Although well-encapsulated, this example appeared to be growing and exhibits foci of central necrosis. (H & E, ×100)

Figure 10—Occult tumors in prostate gland. These have an adenomatous, polyloid character, being covered by normal prostatic epithelium. In this plane of section, the visible portion of the stalk of attachment of the polyloid tumor on the left is very narrow, while that of the tumor on the right is broader. (H & E, ×100)
small T antigens essential for maintenance of the transformed state.\textsuperscript{60} The middle T protein by itself is sufficient to transform cells of some established cell lines.\textsuperscript{61} We conclude that both PTA and RA have normal \textit{ts-a} and \textit{hr-t} gene functions in transformation.

\textit{PTA and RA Show Typical Wild Type Patterns of T Antigen Expression}

Tests for T antigen expression were carried out in infected NIH-3T3 cells. Panel A of Figure 15 shows results of metabolic labeling with \textsuperscript{35}S-methionine followed by extraction, immune precipitation, and polyacrylamide SDS gel electrophoresis. The patterns are indistinguishable. Large (100K), middle (56K), and small (22K) T antigen bands of comparable intensities and mobilities are seen, along with the 63K and 36K “nonviral” T antigen bands, as previously described.\textsuperscript{48} Panel B shows the results of protein kinase assays carried out on immune precipitates labeled \textit{in vitro} with \textgreek{y}-\textsuperscript{32}P-ATP. Most of the incorporation is into a broadly incompletely resolved doublet corresponding to the 56K and 58K species of middle T.\textsuperscript{62}

The band at about 45K represents a proteolytic degra-

\textit{Table 5—Overt and Occult Tumors Induced by the PTA Strains of Polyoma Virus}

<table>
<thead>
<tr>
<th></th>
<th>PTA (uncloned), 32 mice\textsuperscript{†}</th>
<th>PTA (cloned), 37 mice*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Overt</td>
<td>Occult</td>
</tr>
<tr>
<td>Epithelial tumors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hair follicle</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>Thymus</td>
<td>29</td>
<td>2</td>
</tr>
<tr>
<td>Mammary gland</td>
<td>16</td>
<td>3</td>
</tr>
<tr>
<td>Salivary gland</td>
<td>23</td>
<td>4</td>
</tr>
<tr>
<td>Dental epithelium</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Lacrimal gland</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>Thyroid gland</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Adrenal medulla</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Perirethral glands</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td>Bulbourethral glands</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Prostate</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>106</td>
<td>33</td>
</tr>
<tr>
<td>Mesenchymal tumors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subcutaneous sarcoma</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>Hemangiomata</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Bone</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>Renal sarcoma</td>
<td>7</td>
<td>14</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>28</td>
</tr>
</tbody>
</table>

*Data on uncloned virus is from same set of animals as described in Table 2. *Data on cloned virus is from same set of animals as described in Table 3.

\textit{Table 6—Tumor Induction Profile Following Simultaneous Inoculation of PTA and RA Viruses}

<table>
<thead>
<tr>
<th></th>
<th>PTA/RA ((2.5 \times 10^6)/2.5 \times 10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fraction of mice with tumor(s)</td>
</tr>
<tr>
<td>Epithelial tumors</td>
<td>11/11</td>
</tr>
<tr>
<td>Hair follicle</td>
<td></td>
</tr>
<tr>
<td>Thymus</td>
<td>11 (100)</td>
</tr>
<tr>
<td>Mammary gland</td>
<td>3 (27)</td>
</tr>
<tr>
<td>Salivary gland</td>
<td>3 (27)</td>
</tr>
<tr>
<td>Mesenchymal tumors</td>
<td></td>
</tr>
<tr>
<td>Fibrosarcoma (subcutaneous)</td>
<td>1 (9)</td>
</tr>
<tr>
<td>Hemangiomata, any site</td>
<td></td>
</tr>
<tr>
<td>Bone</td>
<td></td>
</tr>
<tr>
<td>Renal medulla</td>
<td></td>
</tr>
</tbody>
</table>

*Numbers in parentheses indicate the percentage of animals in each group with a given tumor.
Table 7 — Assays of Virus Growth and Transformation on Established Fibroblast Cell Lines

A. Single cycle burst sizes on two mouse cell lines*  

<table>
<thead>
<tr>
<th>Virus</th>
<th>Experiment</th>
<th>NIH3T3</th>
<th>UC1-B</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTA</td>
<td>1</td>
<td>1300</td>
<td>940</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>700</td>
<td>770</td>
</tr>
<tr>
<td>RA</td>
<td>1</td>
<td>800</td>
<td>960</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>880</td>
<td>800</td>
</tr>
</tbody>
</table>

B. Transformation of F111 Rat Embryo Fibroblasts†  

<table>
<thead>
<tr>
<th>Virus input</th>
<th>Foci tfu</th>
<th>Efficiency (tfu/pfu)</th>
<th>Agar clones tfu</th>
<th>Efficiency (tfu/pfu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTA</td>
<td>4.8 × 10⁶</td>
<td>7.5 × 10⁶</td>
<td>(1.6 × 10⁻⁴)</td>
<td>2.4 × 10⁻⁷</td>
</tr>
<tr>
<td></td>
<td>2.9 × 10⁶</td>
<td>4.0 × 10⁶</td>
<td>(1.4 × 10⁻⁴)</td>
<td>8.3 × 10⁻⁷</td>
</tr>
<tr>
<td>RA</td>
<td>4.8 × 10⁶</td>
<td>1.0 × 10⁵</td>
<td>(2.0 × 10⁻⁴)</td>
<td>6.0 × 10⁻⁷</td>
</tr>
<tr>
<td></td>
<td>1.1 × 10⁶</td>
<td>1.3 × 10⁶</td>
<td>(1.2 × 10⁻⁷)</td>
<td>9.0 × 10⁻⁷</td>
</tr>
</tbody>
</table>

*Numbers given represent average burst sizes 90 hours after infection.  
†See Materials and Methods.

dation product of middle T. As previously described, this in vitro kinase reaction measures the phosphorylation of the middle T protein by the tyrosine-specific cellular protein kinase, pp60c-src. Polyoma mutants which encode altered middle T proteins that fail to form complexes with pp60c-src are uniformly negative in cell transformation. Both PTA and RA behave as standard wild type viruses in this assay.

The Middle T Protein Encoded by PTA Interacts With pp60c-src in Primary Tumors

The importance of the interaction between the polyoma middle T protein and the product of the cellular proto-oncogene c-src has been documented through a variety of genetic and biochemical experiments carried out in cell culture systems. Polyoma mutants which encode altered middle T proteins that fail to form complexes with pp60c-src are uniformly negative in cell transformation. Both PTA and RA behave as standard wild type viruses in this assay. Measuring pp60c-src tyrosine kinase activity in these complexes, the gel was incubated at 65 C in 1 N NaOH to hydrolyze preferentially phosphoserine and phosphothreonine over phosphotyrosine linkages. Reexposure of the gel showed that the 56-kd and 60-kd phosphoproteins retained their label under these conditions, as is the case with middle T phosphorylated by pp60c-src and autophosphorylated pp60c-src. These results are thus in accord with predictions from studies of polyoma virus in mesenchyme-derived cell culture systems.

Discussion

Molecular biologic studies of the polyoma viruses, particularly mouse polyoma and SV40, have been pursued intensively during the past two decades. Little has been done, however, to relate the results of these studies to infections of the natural hosts. For several reasons, the polyoma-mouse system seems particularly advantageous for such studies. Virus-free inbred mouse strains of different genetic backgrounds are available for study. With appropriate virus and host strains, laboratory infections of newborn mice lead to a wide variety of tumors appearing within a few months. No other oncogen acting in a mammalian system produces a more rapid and divergent pattern of tumor induction. The polyoma-mouse system is also an appropriate model for study of the protective immunity against virus-induced tumors that accompanies natural infections by polyoma viruses (the TSTA response), as well as of mechanisms involved in selective target cell interactions for replication and persistence, all of which are poorly under-
of infection, as well as by the age, genetic background, and immunologic status of the host. From one study to another, and also within single studies, all of these factors have rarely, if ever, been fully controlled, and in no previous tumor-induction study using mice has there been stringent control of genotypic variability within the virus populations administered. A comparative study of virulence (acute lethality) was recently carried out on two plaque-purified virus preparations of polyoma virus although the comparative tumor-inducing activities of those viruses were not reported.¹

In the present study, we have sought to control all of the above factors by administering a uniform dose of virus via a single route (subcutaneously) to mice of a single inbred strain of narrowly restricted age and uniform immunologic status. Most importantly, we administered molecularly cloned, homogeneous virus populations. The results permit us to draw three fundamental conclusions:

1. Polyoma virus of a single genotype can induce neoplasms in at least a dozen different target cell types in mice. The aggregate of these tumor histotypes represents the tumor profile for virus of a specific genotype interacting with hosts of a single genotype and age.

2. Polyoma viruses of dissimilar genotypes can induce widely dissimilar tumor profiles in mice of a single genotype and age, other factors such as dose, diet, and environment being constant.

3. Profound differences in tumor inducing ability are found among virus genotypes having equal transforming ability in culture.

Among the four virus strains surveyed, two general patterns emerged. Highly oncogenic strains PTA and A₃ induced tumors in virtually 100% of injected animals with most recipients developing multiple primary tumors, after a short latency averaging about 3 months. Weakly oncogenic strains RA and A₂ induced predominantly single tumors in only 12–30% of the animals, with an average latency of 10–14 months. An additional striking difference between these two virus groups was in the spectrum of tumor histotypes they induced. The highly oncogenic strains induced multiple tumors of epithelial as well as mesenchymal origin, while the weakly oncogenic strains induced tumors that were uniquely of mesenchymal origin. In animals receiving PTA or A₂, epithelial tumors were the most frequent, the earliest to appear, and posed the greatest threat to the life of the host. Mesenchymal tumors tended to be of much lower frequency and longer latency. The complete absence of epithelial tumors in mice inoculated with RA or A₃.
is remarkable, since the average survival time of these animals was three to four times longer than required for development of such tumors.

The basis for the difference in tropism between the two virus groups is unknown. It is interesting to note, however, that the tissues giving rise to epithelial tumors can be categorized anatomically and developmentally as adnexal derivatives of ectodermal integument (hair follicles, mammary glands, teeth, sweat glands) and of contiguous endodermal integument at either end of the alimentary tract (salivary glands, lacrimal glands, submucosal glands of oropharynx, nasopharynx, and larynx, thyroid, and thymus anteriorly; and prostate, perirectal, and bulbourethral gland posteriorly). The adnexal derivatives of the midpoint of the endodermal integument appear to be exempt (liver, gallbladder, exocrine pancreas, Brunner’s glands, and lungs), although rare examples of polyoma virus-induced primary pulmonary tumors have been reported in mice. It may be relevant that the primary epithelial coats, from which the susceptible adnexal organs are developmentally derived, are also exempt. Specifically, we have not seen tumors arising from epidermis or any of the primary surface-lining epithelia of the alimentary tract (esophagus, stomach, small and large intestines), nor have we seen tumors arising from the surface-lining epithelia of the male or female urogenital tracts. This manner of viewing the polyoma-responsive epithelia suggests that the terminal most highly specialized adnexal derivatives of the primary epithelial coats of ectodermal and endodermal origin are the most sensitive to the oncogenic effects of the virus. This generalization does not account, however, for omissions such as the pituitary and parathyroid glands, preputial and clitoral glands, ceruminous glands, liver, pancreas, and lung.

One remarkable difference in tropism exists between the two highly oncogenic viruses, and this concerns the induction of overt thymic epitheliomas. This tumor was found in 29 of 32 animals inoculated with the PTA strain, but in none of 23 animals receiving virus of the A2 strain. The tropisms of PTA and A2 are otherwise very similar. The singular difference in profiles must relate in some way to differences in the virus genotypes, but its basis is not understood.

Tumor profiles of the highly oncogenic PTA and the weakly oncogenic RA strains were compared after molecular cloning of the viral genomes in an Escherichia coli plasmid vector. The overall profiles were similar in frequency, tropism, and survival time to the profiles obtained with the respective uncloned virus stocks (compare Tables 2 and 3). The high tumor profile of PTA virus stocks therefore appears to be intrinsic to a single virus genotype. Furthermore, the high tumor profile of PTA, including epithelial and mesenchymal tumors, is seen over a 1000-fold reduction of virus dose (Table 4). Apart from a trend toward decreasing frequency of epithelial tumors and increasing frequency of mesenchymal tumors with lower doses of virus, the profiles were otherwise quite similar. The average survival time approximately doubled over the first tenfold dilution. The inverse relationship between dose and survival time suggests
a possible need for replication of the input virus in a low-dose inoculum to attain a tumorigenic threshold range. It is conceivable that susceptibility to tumor induction in different tissues might change over the period of virus amplification and that this would account for the observed shifts in the profile with lower virus dose.

The tumor profiles of cloned and uncloned PTA were also similar when the more rare and occult tumors identified at histologic examination were taken into account (Table 5). While the cloned viruses retained the major features characteristic of the uncloned parental stocks from which they were derived, small but significant differences in the profiles were noted. For example, the cloned PTA virus induced mammary and salivary gland tumors at a somewhat reduced frequency, and subcutaneous fibrosarcomas occurred at a higher frequency than in mice that received the uncloned virus. Similarly, the cloned RA virus seemed to produce a generally lower profile than the uncloned parental stock. It is thus possible that some genotypic heterogeneity capable of affecting tumor profiles exists in uncloned virus preparations.

The results of a single experiment designed to investigate the possibility of a protective effect induced by the weakly oncogenic virus RA were negative. Thus, newborn mice inoculated simultaneously with equal doses of RA and PTA went on to develop a high tumor profile typical of the PTA virus alone (Table 6). This result suggests that the RA virus genotype dictates an intrinsically low transforming and/or replicating capability in the intact host and does not act indirectly by boosting host defense mechanisms.

The results on pathogenicity of PTA and RA demonstrate that the high and low tumor profiles of these strains are due to effects of single virus genotypes in the animal. In an attempt to gain some insight into how the pathogenetic processes induced by these two viruses might differ, the cloned viruses were compared in cell culture systems which permit quantitative measurements of virus growth and cell transformation. Using established mouse and rat cell lines in standard assays, the two viruses behaved indistinguishably (Table 7). Furthermore, their patterns of expression of T (tumor) antigens were the same by metabolic labeling with \(^{35}\text{S}\)-methionine and immunoprecipitation and by formation of complexes between the viral middle T protein and cellular protein pp60\(^{c-src}\) (Figure 15). The expectation that such complexes would be found in primary tumors was borne out by experiments carried out on tumor extracts. Immune precipitations of crude extracts of PTA-induced epitheliomas with anti-T and anti-src antibodies indicated the presence of complexes that are enzymatically active in carrying out tyrosine phosphorylation of middle T by pp60\(^{c-src}\) in vitro (Figure 16).

These comparisons of PTA and RA make it clear that cell transforming ability and expression of middle T:pp60\(^{c-src}\) complexes, though presumably necessary for transformation in the animal, do not by themselves provide a reliable basis for predicting the ability of a particular virus strain to induce tumors in the mouse. Since the comparison of transforming ability in vitro was carried out on rat cells, which are nonpermissive, the possibility exists that the most tumorigenic viral genotypes are simply less cytopidal in mouse cells and therefore a larger proportion of infected cells survive to become transformed. The abilities of high and low tumor virus strains to replicate at various target sites in vivo are now under investigation. Surprisingly, preliminary observations indicate that the most highly oncogenic viruses produce more extensive lytic lesions in organs belonging to the tumor constellation than do viruses of low oncogenicity (T. Dubensky, R. Freund, J. Barncastle, C. Dawe, and T. Benjamin, unpublished).

The mechanisms through which different virus strains induce different tumor profiles remain unknown and of utmost interest. Several plausible and testable hypotheses can be suggested on the basis of currently available information. For example, differences in the noncoding region of the viral genome around the origins of DNA replication may influence the ability of the virus to express its genes in a cell-type specific way.\(^6\) Tissue-specific virus enhancers might operate directly in the potential target cell for tumor induction. Regulatory effects may also occur at the level of virus replication, either in the target tissue or at distant sites, such as kidneys, lungs, and salivary glands, resulting in more or less rapid and intensive dissemination of virus to various target cell sites. An influence of viral regulatory sequences on tumor tropism has been shown in the case of the LTRs (long terminal repeats) of the murine leukemia viruses which affect T-cell lymphomas versus erythroleukemias.\(^7\) Differences in coding sequences are also of obvious potential significance. Differences in the viral capsid proteins, for example, could affect the viruses' ability to adsorb to, enter, and uncoat in different cell types. Similarly, alterations in the structure of the T antigens could affect their manner of interaction with various host cell factors through which they function. The structure of the \(v-abl\) (Abelson murine leukemia virus) gene product provides one example among several that might be cited demonstrating that structural differences in viral oncoreg products affect transforming and tumor-inducing properties of the virus.\(^8\)
By constructing PTA/RA recombinant viruses, involving exchanges of previously defined regulatory and coding sequences, and studying these viruses in animals, it should be possible to pinpoint viral determinants that affect the tumor profile. Additional insights into the molecular aspects of viral pathogenesis in this system should also be possible with the use of specific virus mutants in extensions of the approaches described here.

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