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Polyomavirus Replication in Mice: Influences of VP1 Type and Route of Inoculation

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Patterns of polyomavirus replication and spread have been studied following inoculation of virus into newborn mice. Levels of virus replication in different tissues were followed in situ by using whole mouse section blots and immunoperoxidase staining for the major capsid protein VP1, as well as by tissue extraction and direct quantitation of viral DNA and infectious virus. Patterns of replication and spread were compared between the "high tumor" strain (inducing a high incidence of tumors) PTA and the "low tumor" strain (inducing a low incidence of tumors) RA, following different routes of inoculation. The ability to induce a high tumor profile correlated with the ability to establish disseminated productive infection, with the kidney as a major site of amplification. Furthermore, results with PTA-RA recombinant viruses and site-directed mutants showed that the VP1 specificity of PTA, demonstrated earlier to be a critical determinant for induction of a high tumor profile (R. Freund, A. Calderone, C. J. Dawe, and T. L. Benjamin, J. Virol. 65:335–341, 1991), is also critical for amplification in the kidney and for establishment of disseminated infections.

The original finding that inoculation of the mouse polyomavirus into newborn mice results in the development of multiple tumors is somewhat paradoxical in view of ensuing studies of the virus' interactions with cultured cells (discussed in references 11 and 15). With cells derived from the natural host the interaction is predominantly a lytic one, ending in the death of the cell and production of progeny virus. To study the oncogenic effects of the virus, cells of other rodents, particularly rats and hamsters, have been used. The latter host systems are largely nonpermissive for virus replication; transformed cells are free of infectious virus and typically carry the viral DNA in an integrated state. At the level of the single cell, virus replication and cell transformation are therefore considered to be mutually exclusive outcomes of infection.

Much has been learned at the molecular level concerning the structure, replication, and expression of the viral genome in productive and nonproductive infections by using appropriate cell culture systems. Little is known, however, about how expression of the viral genome is regulated in mice. In particular, the relationship between virus replication and tumor induction remains to be explored. Is tumor induction favored by viruses that are limited in ability to replicate and therefore less cytocidal, or on the contrary, is tumor induction dependent on disseminated infection and attainment of high titers of virus in the animal? If the latter is true, then how is productive (i.e., cytocidal) infection curtailed in those cells destined to become transformed and produce tumors? These questions are particularly compelling because virtually all of the more than a dozen different cell types known to give rise to tumors are also hosts to lytic infection by viruses in the animal (2).

Here we begin to address these questions by following replication of "high tumor" strains (inducing a high incidence of tumors) and "low tumor" strains (inducing a low incidence of tumors) of polyomavirus in newborn mice. The results point clearly to the importance of the VP1 type of the high tumor strain in establishing a disseminated infection and particularly in replicating to high titer in the kidney during the first 2 weeks. A single-amino-acid difference between the VP1s of the high and low tumor strains (5) is therefore critical for productive infection and amplification of virus in the animal as well as for the subsequent development of tumors.

MATERIALS AND METHODS

Virus strains and routes of inoculation. The viruses used in this study have been described in detail elsewhere. Briefly, the wild-type viral strain PTA induces a high incidence of both epithelial and mesenchymal tumors in mice while strain RA induces a low incidence of only mesenchymal tumors (2, 9). The recombinant virus PR-1 contains the noncoding region of PTA and the coding region of RA and is a low tumor strain. RP-3, containing the noncoding region of RA and the coding region of PTA, induces a high tumor profile like PTA (9). Three single-amino-acid differences exist between the coding regions of PTA and RA in middle T, large T, and VP1 (5). The three viruses PTA/RA-mT, PTA/RA-LT, and PTA/RA-VP1 differ from the parental virus PTA by encoding the amino acid present in RA for the indicated protein. Both PTA/RA-mT and PTA/RA-LT induce high tumor profiles, while PTA/RA-VP1 does not (5, 7).

Newborn C3H/BiDa mice (less than 18 h old) were inoculated either subcutaneously with 50 μl, perirenally with 25 μl, or intranasally with 15 μl of a crude virus suspension (titer, 5×10^7 to 5×10^8 PFU/ml). The intranasal inoculation was administered by placing the inoculum over the animals' nostrils and gently squeezing the mouse to stimulate respiratory activity and inhalation of virus. Intrarenal inoculation was attempted by injecting the newborn mouse in the left side through the skin into the visible kidney; however, histological examination indicated that initial infection was confined largely to perirenal tissues. In the text, we therefore refer to these infections as perirenal.

Whole mouse section hybridization. Mice were killed, frozen in OCT, and sectioned parasagittally in a cryostat

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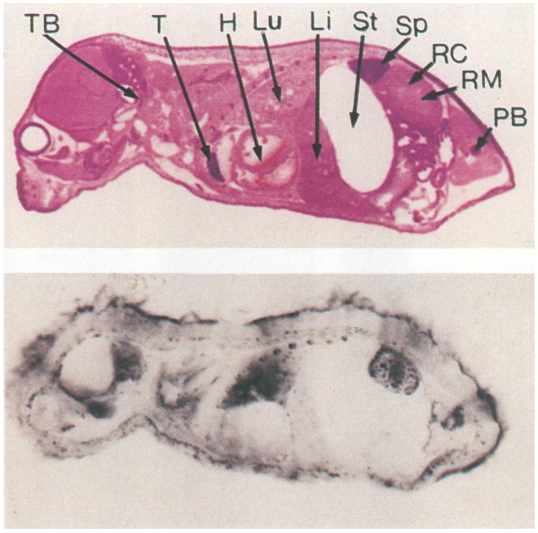

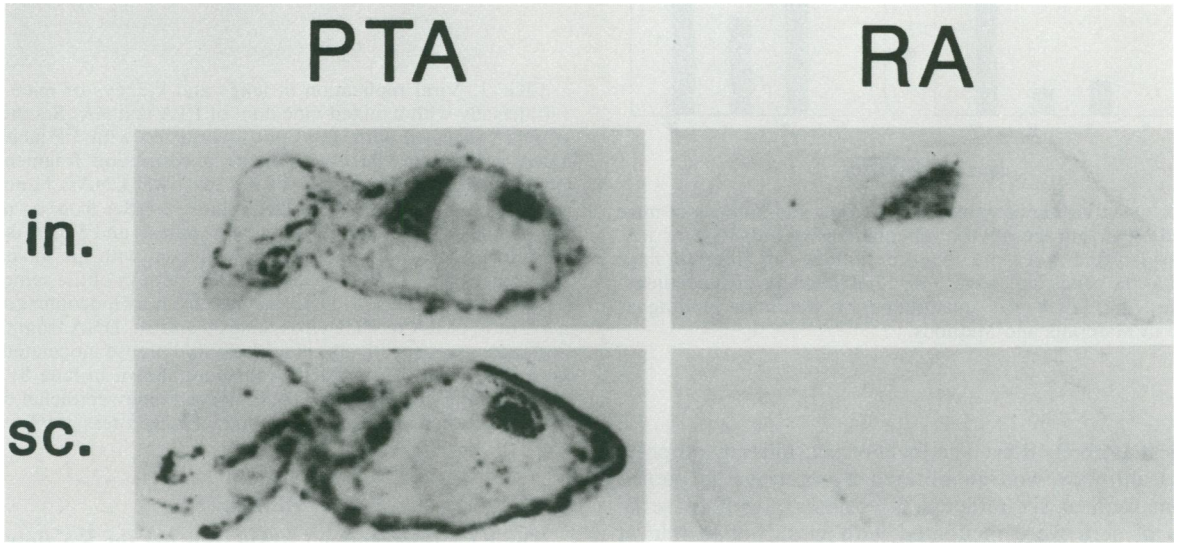

FIG. 1. In situ hybridization to whole mouse sections. (Top) Adjacent parasagittal sections of a 12-day-old mouse infected intranasally with PTA at birth. The top section is stained with hematoxylin and eosin, and the organs are labeled for identification. TB, temporal bone; T, thymus; H, heart; Lu, lung; Li, liver; St, stomach; Sp, spleen; RC, renal cortex; RM, renal medulla; PB, pelvic bone. The adjacent section was hybridized to ^{35}S-labeled polyomavirus DNA, and the autoradiograph is shown. (Bottom) Autoradiographs of whole mouse sections hybridized to ^{35}S-labeled polyomavirus DNA. Mice were inoculated at birth with either PTA or RA intranasally (in.) or subcutaneously (sc.) and killed at 12 days. Exposure time was 12 h.

microtome (Hacker Instruments) at −20°C. One section was transferred to a nitrocellulose membrane; the adjacent section was transferred to a glass microscope slide, fixed in formalin for 1 min, and stained with Harris hematoxylin and eosin. The membrane was hybridized with ^{35}S-labeled nick-translated polyomavirus DNA (specific activity, 5×10^8 cpm/μg), washed, and exposed to Kodak XRP film for autoradiography as previously described (3).

Histological examination and immunocytochemistry. Mice were necropsied at the indicated times postinfection, and the tissues were dissected, fixed in Bouin's fluid, embedded in paraffin, and cut into 5-μm sections. The sections were either stained in standard Harris hematoxylin and eosin or left unstained for immunocytochemistry.

For immunocytochemistry, the peroxidase antiperoxidase procedure (Dako PAP kit) was used with rabbit anti-polyomavirus VP1 as the primary antibody. Positive reaction to the polyomavirus antigen was indicative of productive lytic infection.

Cytologically identified lytic lesions were assigned grades ranging from 0 to +4 on the basis of relative frequency of nuclear changes typical of polyomavirus infection. These changes included nuclear enlargement, often with ballooning, margination of chromatin, presence of Cowdry type A inclusion bodies, and early nuclear disintegration. Grade +1 was arbitrarily defined as an average of approximately one lytic nucleus per ×100 microscopic field, grade +2 was defined as an average of 2 to 5 lytic nuclei per ×100 field, grade +3 was defined as 6 to 10 lytic nuclei per ×100 field (including clusters of lytic nuclei), and grade +4 was defined as any condition more extensive than grade +3. A trace level was assigned to examples with occasional lytic nuclei but below the grade +1 level. This system has some obviously subjective features, but in the hands of a single observer proved to be a reproducible relative assay method.

Recovery of infectious virus. Lungs and kidneys from infected 12-day-old mice were dissected aseptically, weighed, and Dounce homogenized in serum-free Dulbecco's modified medium at a weight-to-volume ratio of 10 mg/ml. The homogenate was incubated for 30 min with 0.1 mg of collagenase per ml at 37°C, the pH was adjusted to 8.0, and the homogenate was then incubated for an additional 30 min at 37°C with 0.1 mg of elastase per ml. The homogenate was frozen and thawed twice and centrifuged to remove cell debris, and the virus in the supernatant was titered by plaque assay (10).

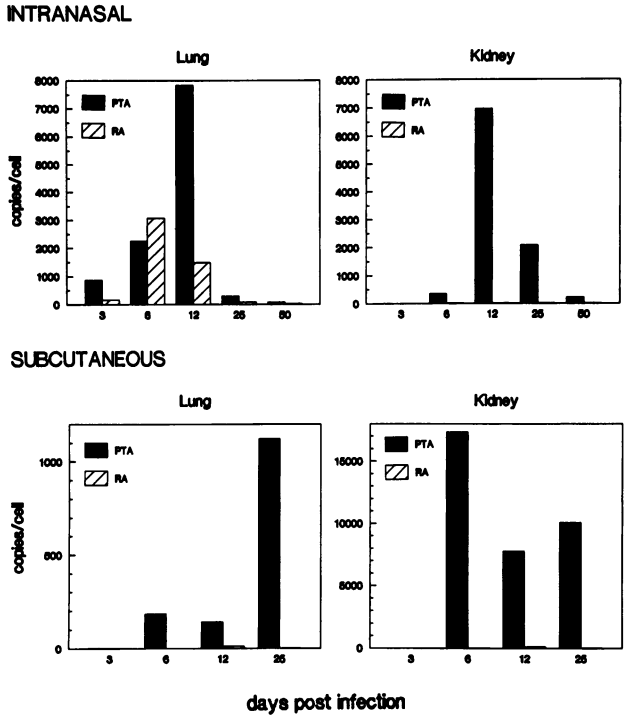

FIG. 2. Quantitation of viral DNA in lungs and kidneys of mice inoculated either intranasally or subcutaneously with PTA or RA. DNA was isolated from the lungs or kidneys of infected mice sacrificed at the times indicated (3, 6, 12, 25, and 50 days postinfection). The viral DNA was quantitated by Southern blotting as described in Materials and Methods.

Antiviral antibody titer. The level of neutralizing antipolyomavirus antibody was quantitated by titering the pooled sera from four to six infected littermates. Twofold serial dilutions of sera were incubated with virus for 30 min at 37°C, and the virus was then titered by plaque assay. Serum titers were calculated as the reciprocal of the highest dilution which gave a 50% reduction in plaque formation. The sera from both PTA- and RA-infected mice neutralized both viruses equally. Sera from uninfected mice did not cause any reduction in plaque formation.

DNA isolation and viral DNA quantification. DNA was extracted from lungs and kidneys of infected mice as previously described (4). To quantitate the viral DNA present in the samples, 10 μg of total DNA was digested with either *Eco*RI or *Msp*I and subjected to electrophoresis on either a 0.8% agarose gel or a 3 to 7% gradient polyacrylamide gel. On each gel, four lanes containing the equivalent of 1, 10, 100, and 1,000 copies per cell of cloned viral DNA were included. The DNA from the agarose gels was transferred to nylon membranes as previously described (13, 16). The DNA from the polyacrylamide gels was electrophoretically transferred to nylon membranes. The membranes were vacuum baked to affix the DNA, prehybridized, probed with ^{32}P-labeled cloned polyomavirus DNA (specific activity, 5×10^8 cpm/μg), and washed as previously described (4). The relative intensities of the signals obtained by autoradiography were determined by densitometric analysis of the film. Viral copy numbers in the DNA samples were obtained by comparison to the reconstructed copy number control lanes.

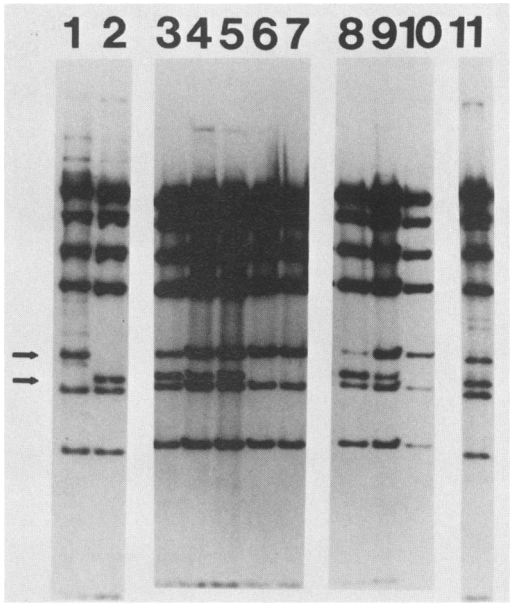

FIG. 3. Viral replication in lungs and kidneys of mice infected intranasally with a mixed inoculum of PTA and RA. Southern blots of DNA digested with *Msp*I and hybridized with ^{32}P-labeled viral DNA are shown. Arrows indicate a restriction fragment length polymorphism in fragment 5 of the two viral DNAs. Lane 1, PTA viral DNA; lane 2, RA viral DNA; lane 3, DNA from an inoculum containing equal titers of PTA and RA; lanes 4 and 5, DNAs isolated from lungs of two 12-day-old mice infected with the mixed inoculum; lanes 6 and 7, DNAs isolated from kidneys of the same mice as in lanes 4 and 5; lane 8, DNA isolated from an inoculum containing a 5:1 ratio of RA to PTA virus; lanes 9 and 10, DNA from the lungs and kidneys, respectively, of a 12-day-old mouse inoculated at birth with the mixed RA and PTA inoculum shown in lane 8; lane 11, DNA isolated from primary baby mouse kidney epithelial cells 48 h after infection with a mixed inoculum of equal titers of PTA and RA.

RESULTS

The PTA (high tumor) strain, but not the RA (low tumor) strain, establishes disseminated infections in the neonatally infected mouse. (i) Whole mouse section blots. Replication of virus was followed by in situ hybridization by using sections of whole mice and ^{35}S-labeled viral DNA probe (see Materials and Methods). Adjacent parasagittal sections were processed by staining with hematoxylin and eosin and by in situ hybridization to identify anatomical sites of replication (Fig. 1, top). Newborn animals inoculated intranasally with the high tumor strain PTA and examined at 12 days showed viral DNA in the lungs, kidneys, skin, and bones, indicating these sites as prominent targets of infection. In comparison, replication of the low tumor strain RA under the same conditions was limited essentially to the lungs. Little or no involvement of the kidney was seen with RA by this method, even after periods of up to 50 days. When the viruses were introduced subcutaneously, the results showed disseminated infection by PTA again involving kidneys, skin, and bones (and to a lesser extent lungs), while RA showed little or no evidence of replication in any tissue (Fig. 1, bottom).

(ii) Quantitative Southern analyses. Quantitative estimates of viral DNA were obtained by DNA extraction from the lungs and kidneys followed by Southern blot analysis. Figure 2 shows results of a time course study based on quantitative Southern blot analyses; averages of estimates from two to four mice are shown for each time in the histograms.

Following intranasal inoculation, PTA achieved maximal levels of replication in the lung and kidney by 12 days, with titers rising first in the lung. RA, under the same conditions, also showed initial replication in the lung over the first 12 days. DNA of both viruses persisted at low levels in the lung throughout the 50-day period. Most noteworthy, however, is the failure of RA to replicate to appreciable levels in the kidney throughout the period of study.

Following subcutaneous inoculation, PTA peaked first in the kidney and later in the lung, with peak levels being at least 10-fold higher in the kidney. RA replication was low in both organs throughout the period of study. The maximal levels of accumulation of viral DNA in the kidney were 50- to 1,000-fold less for RA than for PTA, by either route of inoculation.

(iii) Analysis of virus replication in doubly infected mice. The DNAs of PTA and RA have restriction fragment length polymorphisms in their noncoding regions (6, 9). *Msp*I fragment 5, containing sequences on the early side of the replication origin, can therefore be used to follow the replication of each virus in mice infected with a mixture of the two virus types. Direct comparisons in a single animal by this procedure have the additional advantage of circumventing experimental error due to variations in the efficiency of intranasal infections from animal to animal. Figure 3 shows results of such an experiment involving lungs and kidneys of 12-day-old mice inoculated at birth with mixtures of PTA and RA. Comparisons of lanes 4 and 5 (lungs) with lanes 6 and 7 (kidneys) in Fig. 3 show that only the virus carrying the larger fragment 5 (PTA) is present in the kidneys, while both viruses are present in the lungs. The same discrimination in the kidney is seen when RA is given in a fivefold excess over PTA (Fig. 3, lanes 8 to 10). Interestingly, the high selectivity of the kidney as a site for replication of PTA in the animal is not seen when primary baby mouse kidney cells prepared from 12-day-old mice are infected in culture with a PTA-RA virus mixture (Fig. 3, lane 11).

(iv) Histological examination. The extent of cytolytic lesions in selected organs was assessed histologically and graded on a scale of 0 to +4, as described in Materials and Methods. Although such estimates of cytopathic effect can be expressed only in relative terms for comparative purposes and are somewhat subjective, the results showed good agreement with the data in Fig. 1 and 2. They provide a more complete picture of productive infection than measurements of viral DNA alone and allow identification of particular cell types that undergo productive infection in a wide variety of anatomical sites.

In animals inoculated intranasally with PTA, mean scores for cytolytic lesions at 6, 12, 25, and 50 days were +1, +3.5, +2, and trace, respectively, in the lungs and were 0, +2.5., +2, and +1, respectively, in the kidneys. In mice receiving RA under the same conditions, the lungs showed corresponding scores of +3, +3, +1, and 0, and kidneys showed scores of 0, +1, 0, and 0. Thus, in the lungs, the extent and duration of infection were similar for PTA and RA. Cytolytic lesions were found predominantly in epithelial cells in distal portions of the bronchoalveolar tree. In the kidney, the maximum extent of lytic lesions appeared at 12 days for both viruses but was much lower for RA than for PTA. Lytic lesions in kidneys decreased progressively after 12 days; they were still detectable at 50 days in PTA-infected animals but became undetectable by 25 days in animals inoculated with RA.

A more extensive time course, from 4 to 90 days, was carried out following subcutaneous inoculation of virus. RA induced lytic lesions at extremely low levels, confined to scattered cells of the renal tubular epithelium and bronchial epithelium between 12 and 30 days. In sharp contrast, PTA induced extensive lytic lesions in the kidney by 8 days, mainly in the intertubular mesenchyme of the medulla. At later times, lytic infection by PTA was abundant in the renal tubular epithelium, persisting at high levels through 40 days and declining slowly thereafter. In the lung, PTA induced lytic lesions in bronchial and alveolar epithelum beginning at 13 days and persisting at low levels throughout the 90-day period.

TABLE 1. Recovery of infectious virus from organs of infected mice[a]

Virus	Tissue	Titer recovered (10^3 PFU/mg)
PTA	Lung	91
	Lung	10
	Kidney	45
	Kidney	6
RA	Lung	69
	Lung	37
	Kidney	0.004
	Kidney	0.03

[a] Virus was introduced intranasally into mice less than 18 h old. Animals were killed at 12 days of age, and lungs and kidneys were extracted and assayed for infectious virus (see Materials and Methods).

In addition to the lung and kidney, infection by PTA was noted at multiple secondary sites. These include the skin (first in the dermal mesenchyme, later in the follicular epithelium), thymic epithelium, thyroid follicles, salivary glands, mammary glands, adrenal medulla, walls of the larger arteries (1), mesothelial cells lining body cavities, bones, dental papillae, and in males, glands of the lower urogenital tract and testes (Sertoli cells). In contrast, RA gave no signs of infection at these sites.

Histological examination also revealed microscopic tumors developing in the renal medullary mesenchyme, often bilaterally, as early as 30 days in PTA-infected animals. Primary pulmonary tumors also developed at later times in a fraction of animals inoculated intranasally with PTA (data to be presented in detail elsewhere). Tumors at other sites (mammary glands, salivary glands, thymus, hair follicles) were also evident histologically in PTA-infected animals but not in animals infected with RA, in agreement with earlier studies (2, 6, 7, 9).

(v) Recovery of infectious virus. The failure of RA to spread from the lung to other sites, including the kidney, following intranasal inoculation could be explained if this virus replicated its DNA but failed to carry out a complete productive infection in the lung. To test this possibility, levels of infectious virus recovered from tissues of infected mice were measured by plaque assay on UC1B cells (Table 1). PTA gave comparable titers of infectious virus in the lungs and kidneys. RA grew as well as PTA did in the lungs but failed to grow appreciably in the kidneys. These results on levels of infectious virus parallel those based on estimates of viral DNA (Fig. 2) and of cytolytic lesions and indicate clearly that the lung is a site of amplification for both viruses. Why only PTA and not RA spreads further remains unclear. RA virus particles may either remain trapped in the lung or if

TABLE 2. Progression of renal infection by PTA and RA after perirenal inoculation[a]

Days postinfection	Tissue type containing viral lesions					
	Perirenal connective tissue		Mesenchyme cortical-medullary junction		Epithelium, renal tubules	
	RA	PTA	RA	PTA	RA	PTA
6	++	++	0	0	0	0
10	0	+	+	++++	TR	+
26	0	0	TR	0	TR	+++

[a] Scored on the basis of positive nuclear staining for polyomavirus VP1 by the peroxidase-antiperoxidase method. Scale, 0 to ++++; TR, trace (see Materials and Methods).

they spread to the kidney, be unable to carry out productive infection in that organ. Further experiments addressing this point are described below.

PTA but not RA replicates extensively in the kidney following perirenal inoculation. Results of the preceding experiments indicated extensive infection of the kidney by PTA but not by RA. This could result from unequal abilities of the two viruses either to reach this organ or to infect it once access is gained. The possibility of differential access is raised by the finding that at least in vitro, baby mouse kidney epithelial cells are equally susceptible to the two viruses (Fig. 3, lane 11). To test these possibilities further, virus of each type was inoculated directly into the left kidneys of newborn mice. At approximately 6, 12, and 25 days, animals were killed; both kidneys were removed and examined for productive infection by immunoperoxidase staining for the major capsid protein VP1. Because of the high mortality of animals inoculated perirenally with PTA, this virus was diluted 100-fold and compared with undiluted RA. Results are shown in Table 2 and Fig. 4.

At 6 days postinfection, PTA- and RA-infected animals showed approximately equal numbers of VP1-positive cells, confined largely to the perirenal mesenchyme (at the fatty and areolar connective tissues at the renal hilum, at the upper pole of the kidney near the adrenal gland, and occasionally at the renal capsular cells; [Fig. 4A and D]). At this time, no cells within the kidneys were positive. The perirenal infections were approximately equal in the right and left kidneys for both viruses.

At 10 to 12 days, a striking quantitative difference between the two virus infections became evident. VP1-positive cells were present in the renal medullary mesenchymes with both viruses, but positive cells were much more numerous with PTA than with RA (Fig. 4B and E). With either virus, there was a distinctly zonal distribution of lytic cells within an arciform stratum of mesenchymal cells on the medullary side of the cortical-medullary junction. This stratum of cells is prominent in normal mouse kidneys during the first 2 weeks after birth, when tubulogenesis is still active. In PTA-infected kidneys, this junctional zone of the mesenchyme was so heavily infected that cell necrosis was accompanied by edema and infiltrations of polymorphonuclear leukocytes. In contrast, in the RA-infected kidneys, only isolated small groups of cells in the same stratum of the mesenchyme were VP1 positive and there was no appreciable leukocyte response. With PTA, but not with RA, there was also some extension of infection into the renal cortex, mainly involving the intertubular mesenchyme but occasionally involving the tubular epithelium as well.

At 26 to 27 days postinfection, a third stage of infection emerged. For both viruses, the cell type infected was predominantly the renal tubular epithelium, but the number of epithelial cells infected was obviously far greater for PTA than for RA (Fig. 4C and F). Lytically infected epithelia were found in proximal and distal convoluted tubules and in collecting tubules as well. In PTA-infected kidneys, VP1-positive cells were sometimes seen in the parietal layer of Bowman's capsule in glomeruli and also occasionally in urothelium lining the renal papilla and pelvis. With PTA, nearly every microscopic field (×100) contained many infected tubules, whereas with RA, only a few infected tubules were noted scattered amid wide expanses of uninfected tubules.

Summarizing, lytic infection, as reflected by the presence of VP1, appeared first in perirenal and capsular connective tissues and then proceeded to involve the mesenchymal cells of the medulla at the cortical-medullary junction and ultimately the tubular epithelium at all levels of the nephron. The first stage of infection (perirenal) was approximately equally extensive in PTA- and RA-infected mice, while the two later stages (intrarenal) were much more extensive in PTA-infected mice. The differential ability of the two viruses to establish intrarenal infection is underscored by the fact that the dose of RA was approximately 100-fold greater than that of PTA.

At each time, the pattern and extent of infection were similar in the left (directly inoculated) and right (uninoculated) kidneys of individual animals. This suggests the likelihood that virus spread retroperitoneally to reach the right kidney at the time of inoculation.

VP1 of PTA is required for efficient replication in the kidney and establishment of disseminated infections. Exchanges of DNA fragments between PTA and RA involving all or part of their noncoding regions had little or no effect on the tumor profiles (9), with one exception (6). These results indicate the existence of one or more structural determinants in PTA that are essential for induction of a high tumor profile. A further analysis based on complete sequences of the two viral DNAs has shown that a single-amino-acid substitution in VP1 is a major determinant for induction of tumors, particularly those of epithelial cell origin (5). The following experiments were carried out to see to what extent the viral genetic determinants for replication in mice follow those established for tumor induction.

Figure 5 shows the results of exchanging the noncoding regions between PTA and RA. Mice were infected with a 1:1 mixture of the recombinant virus strains designated PR-3 and RP-1. PR-3 is a low tumor strain that derives all of its noncoding sequences from PTA and all of its coding sequences from RA; RP-1, a high tumor strain, is the reciprocal recombinant (9). With this mixture of viruses, it is RP-1 carrying the smaller fragment 5 which replicates in both the lung and kidney, while PR-3 is confined to the lung (compare Fig. 3, lanes 4 through 7, with Fig. 5, lanes 4 through 7). Thus, as in studies of tumor-inducing ability by these same recombinant strains, the ability to spread and replicate in the kidney requires the coding regions of PTA. A comparison of the amount of replication of PTA (Fig. 3) and RP-1 (Fig. 5) suggests that the noncoding region contributes to the level of replication in the kidney. This difference may also be reflected in the tumor profiles of these viruses (9).

Oligonucleotide mutagenesis was used to introduce the RA coding specificities for large T, middle (and small) T, and VP1, separately, into the background of PTA. PTA bearing either or both of the T-antigen specificities of RA retained

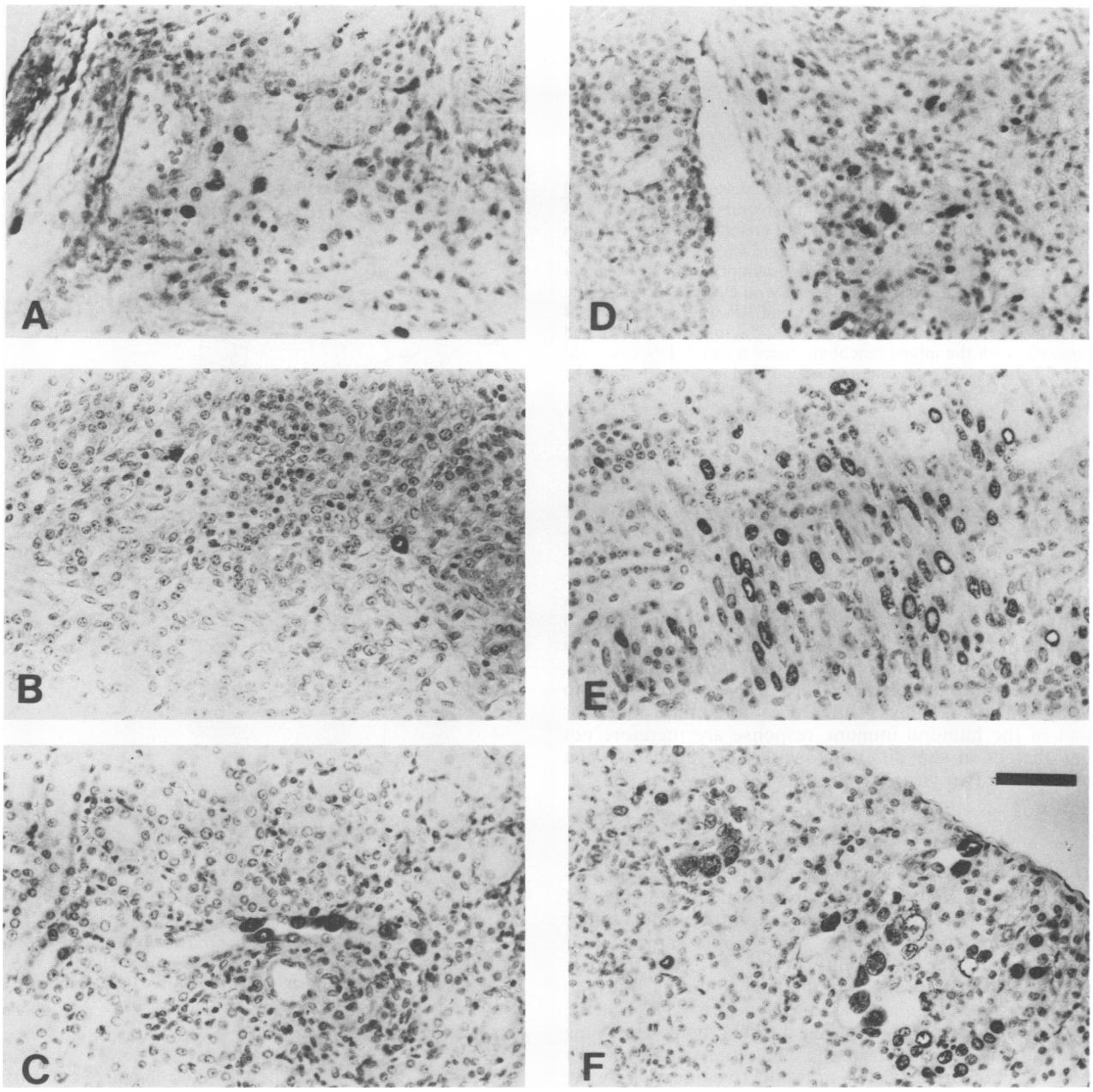

FIG. 4. Lytic lesions in kidneys after perirenal inoculation with virus strains PTA and RA. The cells in panels A, B, and C are from mice that were recipients of RA at 6, 10, and 26 days postinoculation, respectively. The cells in panels D, E, and F are from mice that were recipients of PTA at the same times. All sections are stained for VP1 by the peroxidase-antiperoxidase method; dark-staining nuclei are positive by this method. At 6 days (A and D), positive cells are found only in perirenal connective tissue and are equally numerous for the two viruses. At 10 days, only occasional mesenchymal cells are positive in a zone at the cortical-medullary junction of the RA-inoculated kidney (B), while many mesenchymal cells in the same zone are positive in the PTA-inoculated kidney (E). At 26 days, occasional renal cortical tubules show positive epithelium after RA inoculation (C), while positive tubular epithelial cells are very numerous after PTA inoculation (F). The magnification of all the micrographs is the same as shown in panel F. The bar represents 50 nm.

the ability to induce a high tumor profile. However, introduction of the RA VP1 specificity resulted in a dramatic decrease in tumor induction (5, 7). These same mutants were examined for their replication patterns by the whole mouse section blot procedure. The results shown in Fig. 6 demonstrate that the ability to reach and replicate in the kidney following intranasal inoculation depends on retention of the PTA VP1 specificity. Introduction of the single-amino-acid substitution of RA VP1 (i.e., glycine instead of glutamic acid at position 92) is sufficient to prevent or severely limit the appearance of virus in the kidney.

Antiviral immune responses are approximately the same in PTA- and RA-infected tissue. The decrease in virus titers beginning around 12 days corresponds to the development of humoral immunity (12). The initial rise and early disappearance of RA in the animal could indicate a role of the immune

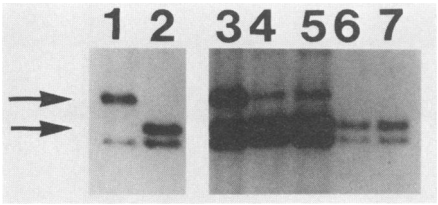

FIG. 5. Viral replication in lungs and kidneys of mice inoculated intranasally with a mixed inoculum of the recombinant viruses PR-3 and RP-1. A portion of the Southern blot containing DNA cut with *Msp*I and hybridized with ^{32}P-labeled viral DNA is shown. Arrows indicate the restriction fragment length polymorphisms of fragment 5. Lane 1, Viral DNA from PR-3; lane 2, viral DNA from RP-1; lane 3, DNA isolated from the mixed PR-1 and RP-3 inoculum; lanes 4 and 5, DNA isolated from the lungs of 12-day-old mice inoculated intranasally with the mixed inoculum; lanes 6 and 7, DNA isolated from the kidneys of 12-day-old mice inoculated intranasally with the mixed inoculum.

response in limiting spread and infection by this virus. That antiviral antibody could differentially affect the two viruses would appear unlikely in view of the results with doubly infected mice (Fig. 3). Nevertheless, development of neutralizing antibody was monitored in animals inoculated intranasally with PTA or RA. Titers of sera from infected mice were determined by serial dilution and neutralization of virus infectivity in a plaque assay, as described in Materials and Methods. Mice infected with either virus responded with a similar time course and with titers of similar magnitude. Titers were approximately 1,000 at 12 days and increased to roughly 10,000 at 25 days. Differences in the time course or extent of the humoral immune response are therefore not major factors in the different degrees of replication and spread by the two viruses.

DISCUSSION

The relationship between polyomavirus replication in neonatally infected mice and the subsequent development of tumors has been investigated by using prototype high tumor and low tumor strains of virus. The results show a clear correlation between the ability of the virus to replicate efficiently and to spread within the animal and its ability to induce tumors. Although the kidney is the organ showing the highest levels of virus replication, the skin, bones, and lung and to varying degrees, all tissues that compose targets for tumor induction are also sites of productive infection by the high tumor strain of virus. In the kidney, peak levels of replication by the low tumor strain were lower than those achieved by the high tumor strain by several orders of magnitude. While virus of both types replicated equally well in the lung following intranasal inoculation, at every other site and for all routes of inoculation, the low tumor strain either failed to replicate to a detectable level or did so far less efficiently than the high tumor strain did.

The marked differences in biological properties between the PTA and RA virus strains rest largely on a single genetic difference leading to a single-amino-acid substitution in the major capsid protein (5). The substitution of glycine (RA) for glutamic acid (PTA) at position 92 of VP1 renders the virus defective in the animal with respect to both virus replication and tumor induction. Interestingly, this difference in VP1 has little or no effect on virus replication or cell transformation in culture (2). The simplest and most straightforward interpretation of these findings is that this polymorphism in

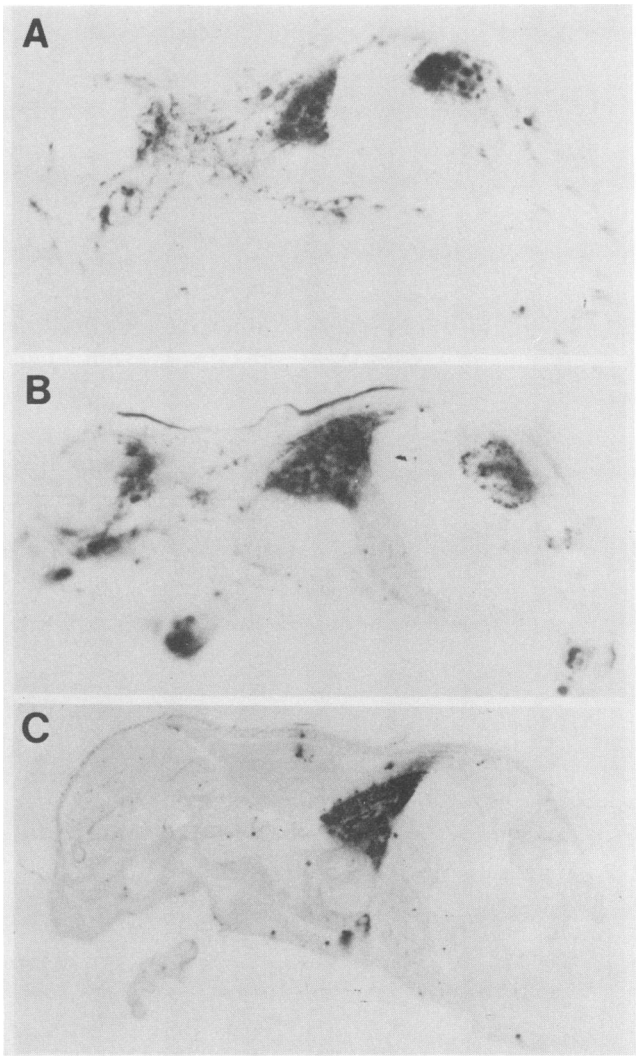

FIG. 6. In situ hybridization to whole mouse sections of 12-day-old mice inoculated intranasally at birth with the recombinant viruses PTA/RA-mT (A), PTA/RA-LT (B), and PTA/RA-VP1 (C). Hybridization was done with ^{35}S-labeled viral DNA. Exposure time was 12 h.

VP1 affects the ability of the virus to recognize cell surface receptors. Additionally, it must be assumed that receptors on certain cells in the mouse differ from those expressed on mouse and rat fibroblasts in culture in such a way as to allow discrimination between the two VP1 types. Direct evidence of the importance of the VP1 change in receptor recognition is presented in an accompanying report (8). While the present results establish the VP1 type as a critical determinant for both replication and tumor induction, they do not necessarily mean that extensive replication is by itself sufficient for induction of a high tumor profile. Additional viral genetic factors could operate to affect tumorigenesis. Mutations in the middle T oncogene, for example, can have both qualitative and quantitative effects on the tumor profile (14).

Discrimination at the level of virus-receptor interaction could operate either directly in target cells destined to be transformed and to grow into tumors or in cells in which the virus must replicate in order to gain access to those target cells or both. The finding that PTA induces tumors of both

epithelial and mesenchymal origin while RA induces tumors only of mesenchymal origin suggests the possibility of discrimination at the level of receptors expressed by these two broad classes of target cell types (2, 5, 9). Results reported here on patterns of virus replication and spread suggest another possibility, namely, that receptor discrimination may also occur at the level of the vascular endothelium. Following intranasal inoculation, both PTA and RA gain direct access to the lung and replicate well at that site. However, only PTA disseminates further to a wide variety of distant sites, a pattern of spread that must occur by the vascular route. Following subcutaneous or perirenal inoculation, PTA also spreads rapidly throughout the animal, while RA replication is confined largely to the site of inoculation. Polyomavirus is known to induce lytic lesions as well as tumors of the vascular endothelium, particularly those strains carrying the PTA-VP1 type (1). Thus, interactions with the vascular endothelium, allowing virus to gain access to and egress from the circulatory system, may be an important factor requiring the PTA type of VP1.

The finding of a positive correlation between virus replication and tumor induction would not be surprising were it not for our understanding of the basic dichotomy between productive and nonproductive infections. In the former case, the complete virus growth cycle is accompanied by cell death; in the latter, virus growth is shut down, allowing the cell to survive and emerge as a transformed cell. This understanding has been based largely on studies of virus-cell interactions by using cultured cells from heterologous species as well as from the natural host (15). The present results thus raise numerous questions concerning regulation of responses to the virus at the cellular level in the intact host. Investigations of the state of the viral genome and regulation of its expression in tumor cells should provide some insights into these unresolved questions.

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REFERENCES

1. **Dawe, C. J., R. Freund, J. P. Barncastle, T. W. Dubensky, G. Mandel, and T. L. Benjamin.** 1987. Necrotizing arterial lesions in mice bearing tumors induced by polyoma virus. J. Exp. Pathol. **3**:177–201.
2. **Dawe, C. J., R. Freund, G. Mandel, K. Ballmer-Hofer, D. T. Talmage, and T. L. Benjamin.** 1987. Variations in polyoma virus genotype in relation to tumor induction in mice: characterization of wild type strains with widely differing tumor profiles. Am. J. Pathol. **127**:243–261.
3. **Dubensky, T. W., F. A. Murphy, and L. P. Villarreal.** 1984. The detection of DNA and RNA virus genomes in the organ systems of whole mice: patterns of mouse organ infection by polyomavirus. J. Virol. **50**:779–783.
4. **Dubensky, T. W., and L. P. Villarreal.** 1984. The primary site of replication alters the eventual site of persistant infection by polyomavirus in mice. J. Virol. **50**:541–546.
5. **Freund, R., A. Calderone, C. J. Dawe, and T. L. Benjamin.** 1991. Polyomavirus tumor induction in mice: effects of polymorphisms of VP1 and large T antigen. J. Virol. **65**:335–341.
6. **Freund, R., C. J. Dawe, and T. L. Benjamin.** 1988. Duplication of noncoding sequences in polyomavirus specifically augments the development of thymic tumors in mice. J. Virol. **62**:3896–3899.
7. **Freund, R., C. J. Dawe, and T. L. Benjamin.** 1988. The middle T proteins of high and low tumor strains of polyomavirus function equivalently in tumor induction. Virology **167**:657–659.
8. **Freund, R., R. L. Garcea, R. Sahli, and T. L. Benjamin.** 1991. A single-amino-acid substitution in polyomavirus VP1 correlates with plaque size and hemagglutination behavior. J. Virol. **65**: 350–355.
9. **Freund, R., G. Mandel, G. G. Carmichael, J. P. Barncastle, C. J. Dawe, and T. L. Benjamin.** 1987. Polyomavirus tumor induction in mice: influences of viral coding and noncoding sequences on tumor profiles. J. Virol. **61**:2232–2239.
10. **Goldman, E., and T. L. Benjamin.** 1975. Analysis of host range of non-transforming polyomavirus mutants. Virology **66**:372–384.
11. **Gross, L.** 1983. The polyoma virus, p. 737–828. *In* Oncogenic viruses. Pergamon Press, Oxford.
12. **Rowe, W. P., J. W. Hartley, J. D. Estes, and R. J. Huebner.** 1959. Studies of mouse polyoma virus infection. I. Procedures for quantitation and detection of virus. J. Exp. Med. **109**:379–391.
13. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
14. **Talmage, D. T., R. Freund, A. T. Young, J. Dahl, C. J. Dawe, and T. L. Benjamin.** 1989. Phosphorylation of middle T by $pp60^{c\text{-}src}$: a switch for binding of phosphatidylinositol 3-kinase and optimal tumorigenesis. Cell **59**:55–65.
15. **Tooze, J.** 1980. DNA tumor viruses, p. 125–295. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Wahl, G. M., M. Stern, and G. R. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization using dextran sulfate. Proc. Natl. Acad. Sci. USA **76**:3683–3687.