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The Primary Site of Replication Alters the Eventual Site of Persistent Infection by Polyomavirus in Mice

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Using DNA blot analysis, we monitored the course of polyomavirus infection in mice receiving an intranasal inoculation and compared this with the course of infection in mice receiving an intraperitoneal inoculation. Intranasal infection was characterized by an initial primary replication phase in the respiratory tract, followed by a systemic infection of the visceral organs. At 12 days postinfection, there was partial clearing of viral DNA in all organs; by 22 days postinfection, viral DNA persisted only in the lungs and kidneys, and the level of DNA slowly decreased during the next 3 months. Lungs have been a previously unrecognized site for polyomavirus persistent infection. In contrast to intranasal infection, intraperitoneal infection of mice was characterized by only three phases: an initial systemic phase in which viral DNA was found in the same respiratory and visceral organs as during intranasal infection, clearing of the virus from the organs, and ultimately, a persistent infection in the kidneys but not in the lungs. Thus, different organs became persistently infected when mice were inoculated via these different routes.

Polyomavirus is known to persistently infect the kidneys of mice (9, 16). The duration of this infection can be for the lifetime of the animal when naive newborn mice are infected. It is assumed that a persistent infection occurs after a systemic infection which has followed an initial respiratory infection. Infection via the respiratory tract seems likely, as substantially higher doses are required to infect through the stomach (14, 16). Previous studies of the pattern of organ infection in newborn mice by polyomavirus have been with intraperitoneal (i.p.) inoculation (7–11, 15, 16). Polyomavirus is highly oncogenic when inoculated i.p. into newborn mice (19) but polyomavirus-induced tumors rarely appear in mice inoculated intranasally (i.n.) or acquiring infection naturally (7, 14). It was of concern that i.p. virus inoculation alters the normal pattern of organ infection. In other systems, the immune response of the host has been to vary with the route of virus inoculation (1, 17). When mice are infected via i.p. inoculation with polyomavirus, primary replication of virus in the upper respiratory tract organs is circumvented, thereby avoiding the normal spread of virus from the primary organs to secondary systemic replication sites. It is clear that polyoma persistent infection in mice involves a dynamic relationship between virus and host, and this relationship may be altered by differing the routes of virus inoculation. To study the normal course of events which lead to polyomavirus persistent infections, we have infected mice by a route of infection which most likely occurs in nature (T. W. Dubensky, F. A. Murphy, and L. P. Villarreal, J. Virol., in press).

We have followed by DNA blot analysis the course of polyomavirus infection in newborn mice receiving an i.n. inoculation leading to a persistent infection and compared this with the pattern observed after i.p. inoculation. We show that although the same organs were initially infected, the levels of virus replication and the eventual organs of persistent infection differ with the route of inoculation.

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MATERIALS AND METHODS

Cells, viruses, and inoculations. The protocol for polyomavirus propogation on mouse 3T6 cells has been described by others (4). Cells were grown in the presence of 7% fetal calf serum and nonessential amino acids in Dulbecco medium and maintained in 2% fetal calf serum during virus infection. Cells were infected with a multiplicity of infection of 0.01 PFU of polyomavirus A2 per cell. Extensive cytopathic effects generally occurred within 2 weeks, at which time the cells were scraped from the plate with a rubber spatula, recovered by low-speed centrifugation (2,000 rpm, 20 min), resuspended in medium (1 ml per 100-mm plate), and sonicated. The medium supernatant was saved. The cell debris was then removed by low-speed centrifugation. Plaque assays were performed on 3T6 cells overlaid with 0.8% agarose (Bethesda Research Laboratories) and maintenance medium, as previously described (12). Titers of 10^{10} PFU/ml were normally obtained from the infected sonicated cells; titers of about 10^8 were obtained from the supernatant. BALB/c mice were inoculated either i.n. or i.p. with 10^8 PFU of polyomavirus (ca. 10 μl) within 24 h of birth.

Virus and antibody assays. For detection of viremia, mice were exsanguinated, and the blood was divided into two portions. One portion was heparinized; the other portion was allowed to coagulate, and the serum was then recovered after centrifugation (microfuge, 5 min). Dilutions of heparinized whole blood or serum were then allowed to adsorb onto 3T6 cells for 1 h; they were than overlaid with agarose and maintenance medium as described above. Plaques were detected at 2 weeks. The measurement of anti-polyomavirus antibody was done by serum plaque reduction (17). Serum was isolated as described above. Endogenous virus was inactivated by UV irradiation for 30 min. Dilutions of serum were then incubated with 50 PFU of polyomavirus for 30 min at 37°C and then adsorbed onto 3T6 cells and overlaid, as described above. The endpoint was calculated as the highest dilution of serum giving a 50% reduction in plaque formation.

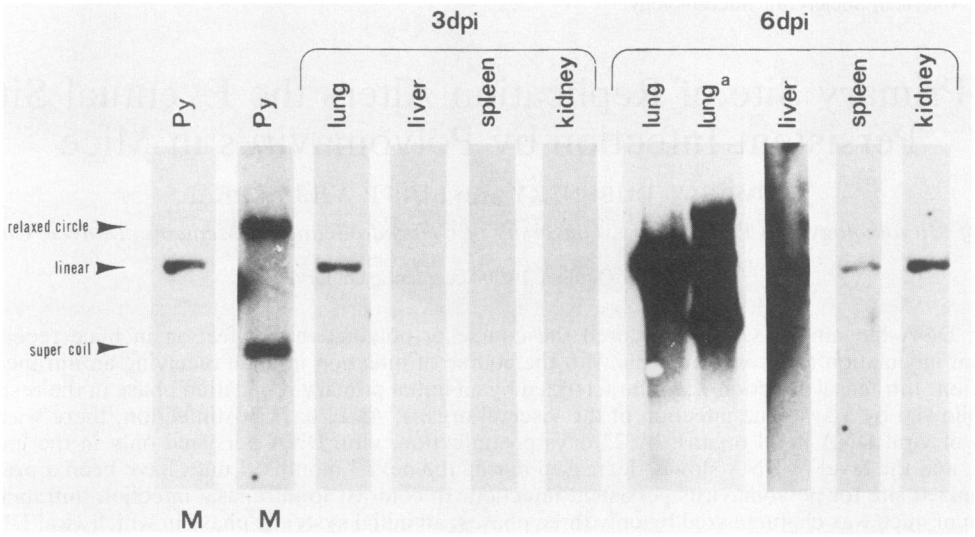

FIG. 1. DNA blot hybridization with extracted tissue DNA from i.n. polyomavirus-infected mice at 3 and 6 dpi. Newborn mice were infected as described in the text. The polyomavirus marker lanes are labeled M. DNA of all tracks (except the track labeled lung[a]) were digested with *Bam*HI. Exposure was for 24 h.

Southern blot analysis of tissue DNA. Total tissue DNA was extracted from the organs and at times as indicated in the figure legends by using modifications of techniques described previously (9). Nuclei were first isolated from homogenized tissue in reticulocyte standard buffer (0.25 M sucrose, 25 mM NaCl, 5 mM $MgCl_2$, 10 mM Tris, pH 7.5) with 0.2% Nonidet P-40. Nuclei were then treated for 1 h each with RNase A at a concentration of 10 μg/ml at 37°C and then proteinase K at a concentration of 100 μg/ml at 50°C. This was followed by extraction with phenol-chloroform (1:1, water saturated). The aqueous phase was dialyzed extensively against Tris-EDTA buffer and concentrated within the dialysis tubing by covering it with dry polyethylene glycol (no. 8000; J. T. Baker Chemical Co.). Approximately 10 μg of tissue DNA was digested with *Bam*HI (cuts polyomavirus DNA once) according to the conditions of the supplier and loaded on a horizontal 1% agarose gel. To determine viral DNA copy number per cell, polyomavirus DNA was isolated from 3T6 cells as previously described (6) and purified on CsCl gradients. Virus copy number reconstructions were performed as follows. DNA from 2×10^6 3T6 cells was extracted and quantitated to be 10 μg. Purified viral DNA (11.6 pg) was added to 10 μg of calf thymus DNA. This is equivalent to one copy of polyomavirus DNA per 2×10^6 cells. This mixed DNA was then run on a gel as a marker lane. Gels were run in Tris-borate-EDTA buffer (per liter: 10.8 g of Tris base, 5.5 g of boric acid, 0.93 g of EDTA) and blotted to nitrocellulose as described previously (18). The filters were air dried and baked for 2 h at 80°C in a vacuum. Both prehybridization and hybridization were for 24 h at 45°C in 50% formamide, 5× SSC (1× SSC: 0.15 M NaCl plus 0.015 M sodium citrate), 10% dextran sulfate, 0.3% sodium dodecyl sulfate, 2× Denhardt solution (3) (1× Denhardt solution: 0.02% bovine serum, 0.02% Ficoll, 0.02% polyvinylpyrrolidone), and 200 μg of calf thymus DNA per ml. In addition, the hybridization solution included 10^6 cpm per transferred gel lane of ^{32}P-labeled nick-translated (13) polyomavirus DNA cloned into pBR322 at the *Bam*HI site with a minimum specific activity of 10^8 cpm/μg of DNA. Washing conditions were as follows: four times in 2× SSC with 0.1% sodium dodecyl sulfate for 5 min at room temperature, followed by two times in 0.1× SSC–0.1% sodium dodecyl sulfate for 30 min at 50°C. Dried filters were exposed to Kodak XAR-5 film at −70°C in the presence of a Lightning Plus intensifying screen (Du Pont Co.) for the times indicated in the figure legends.

RESULTS

i.n. infection of newborn mice. Primary and secondary replication of polyomavirus DNA was determined by DNA blot analysis of various organs at 3 and 6 days postinfection (dpi) (Fig. 1). Viral DNA is found predominantly in the lungs during primary replication at 3 dpi, followed by a general systemic infection involving the liver, spleen, and kidneys by 6 dpi. The 6-dpi panel was overexposed to allow direct comparison to the 3-dpi lanes and so that the viral DNA in the visceral organs could be visualized. As this DNA was extracted from isolated nuclei, it is likely that most of the polyomavirus DNA detected is within the nucleus. The quantity of viral DNA in the lungs at 6 dpi is shown more clearly in Fig. 3 (discussed below). Polyomavirus DNA was present as a free, supercoiled molecule as shown in undigested lung DNA at 6 dpi. No high-molecular-weight viral DNA was seen in the undigested lung DNA. Little, if any, integration of viral DNA into chromosomal DNA occurred. Because equal amounts of DNA were loaded onto each lane, the large increase in signal intensity in the lungs at 6 dpi over that at 3 dpi indicated ongoing viral DNA replication. Partial clearing of viral DNA from all infected organs occurred by 12 dpi (Fig. 2). There was about a 10-fold decrease of viral DNA in the lungs, liver, and kidneys compared with amounts at 6 dpi; viral DNA was not detected in the spleen at this time and was not detected in the brain at any time (data not shown).

Eventually, persistent infections were established only in the lungs and kidneys after i.n. inoculation (shown below). We therefore accurately quantitated the level of viral DNA present in these two organs during the primary, systemic, and clearing phases of infection (3, 6, and 12 dpi, respectively) (Fig. 3). At 3 dpi, ca. 1 copy of polyomavirus DNA per cell was present in the lung. At 6 dpi, ca. 1,000 copies of polyomavirus DNA per cell were present in the lung. Al-

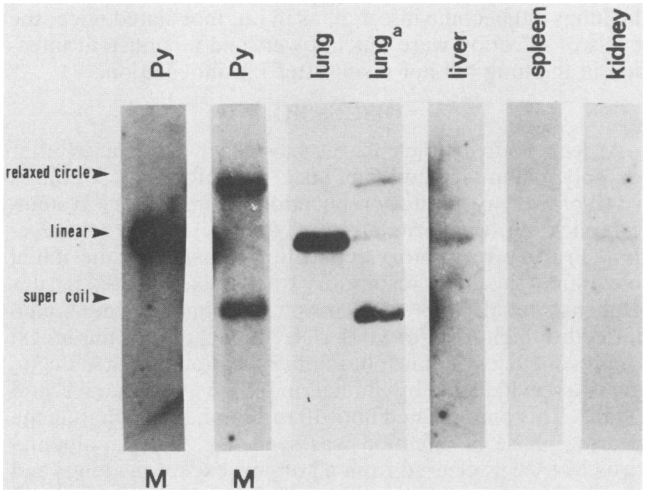

FIG. 2. DNA blot hybridization with extracted tissue DNA from i.n. polyomavirus-infected mice at 12 dpi. Symbols and exposure are the same as in the legend to Fig. 1.

though viral DNA replication level in the lung at 6 dpi was very high, histological examination showed little necrotic lung tissue; hyperplastic cells, however, were seen (unpublished data). During the clearing phase of infection at 12 dpi in the lung, the viral DNA band was present at about 200 copies per cell. At 12 dpi, viral DNA was cleared from the kidney to a level of 1 copy per cell, compared with 10 copies per cell of polyomavirus DNA present during the systemic infection phase at 6 dpi. Viral DNA is not detected in the kidney at 3 dpi.

Levels of viral DNA present in the lung and kidney during long-term infection are shown in Fig. 4. During this phase of persistent infection, viral DNA existed only as a free, supercoiled molecule in the lungs and kidneys. No high-molecular-weight or defective viral DNA was detected. Titration experiments to determine the sensitivity of our blot hybridizations were performed as described above. We were able to detect 1 viral genome copy per 100 cells, using 10 μg of cellular DNA. Therefore, if integrated or defective viral DNA was present, it was at levels lower than this. The level of viral DNA was greater in the lungs than the kidneys at both 22 and 84 dpi. The quantity of polyomavirus DNA in the lungs at 22 dpi was estimated to be between 1 and 10 copies per cell, decreasing to about 1 copy per cell by 84 dpi (Fig. 4). Viral DNA persisted in the kidney at about 1 copy per cell at 22 dpi and decreased to less than 1 copy per cell by 84 dpi. Thus, polyomavirus was seen to persist in the lungs after i.n. inoculation at about 10-fold-higher levels than in the kidneys. Polyomavirus persistent infection in the lungs has not been previously reported.

Viremia and viral antibody development after i.n. infection. The time course of the onset of viremia and the development of viral antibody was followed in i.n. infected mice to determine whether the development of humoral immunity coincided with the pattern of viral replication in the various organ systems, as detected by the Southern blot analyses. These data have been previously reported for mice infected by i.p. inoculation (16). As an i.n. infection led to a different pattern of persistent infection, it was important to determine whether the kinetics of the immune response were different by this route. An inverse relationship existed between viremia and polyomavirus-neutralizing antibody (Fig. 5). Viral titers were determined in both whole blood and serum to establish whether the virus was cell associated. Levels of viremia in whole blood and serum are similar, suggesting that polyomavirus is free in the serum. However, during primary replication at 3 dpi, the virus titer in serum was 5×10^3 PFU/ml compared with a titer of ca. 1×10^2 PFU/ml in whole blood. Virus titers were highest at 6 dpi, coinciding well with the pattern of the widespread tissue involvement as shown by DNA hybridizations at 6 dpi. The onset of viral antibody coincides with the decrease in infectious virus in the blood, reaching a titer of about 1:500 at 15 dpi. Thus, the decrease in virus titer and in quantities of viral DNA coincide with the onset of viral antibody. In previous studies with i.p. infected newborn mice, viremia levels in the serum and whole blood were similar to the viremia levels in i.n. infected mice during the acute phase of infection (16).

i.p. infection of newborn mice. The preceding results indicated that polyoma could establish a persistent infection

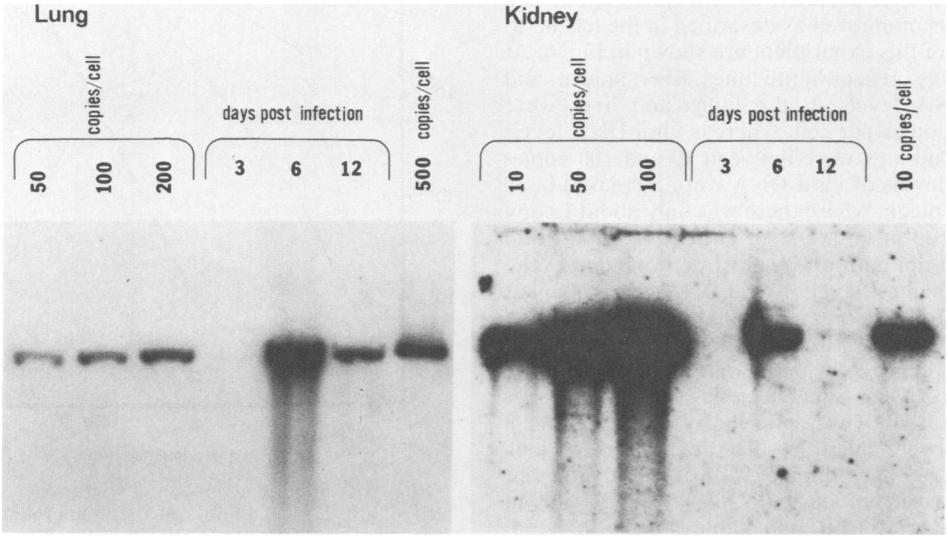

FIG. 3. Quantitation of viral DNA in lung and kidney from i.n. polyomavirus-infected mice at 3, 6, and 12 dpi. DNA (10 μg) was loaded on tissue tracks. Copy number per cell reconstruction tracks were based on 10 μg of cellular DNA (2×10^6 cells). All tracks were digested with *Bam*HI. Exposure times were 12 h for the lung panel and 1 week for the kidney panel.

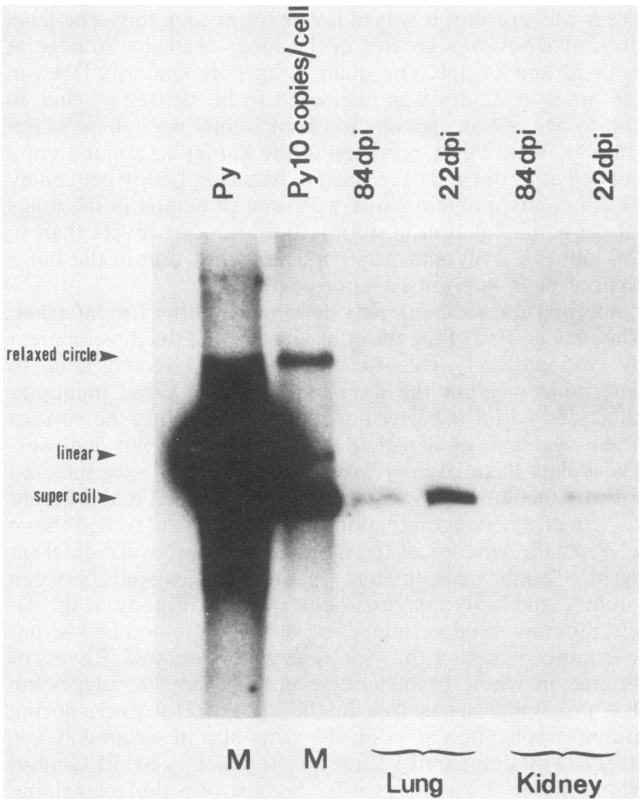

FIG. 4. DNA blot hybridization with extracted lung and kidney DNA from i.n. polyomavirus-infected mice at 22 and 84 dpi. Undigested DNA was loaded on tissue tracks. The polyomavirus marker lanes are labeled M. Supercoiled polyomavirus DNA from 3T6 cells was used for the reconstruction track. Exposure time was 1 week.

in the lungs when inoculated i.n. As this observation was not made during previous studies with i.p. infected mice, it was important to establish that this difference was not due to differences in the virus or mouse strains which we had used. To show this, mice were infected by i.p. inoculation, and viral replication was monitored as described in the legend to Fig. 1. The results of this experiment are shown in Fig. 6. At 3 dpi, viral DNA was present in the lungs, liver, spleen, and kidneys. Viral DNA levels in the lungs and liver were between 1 and 10 copies per cell, whereas viral DNA levels in the spleen and kidneys were between 10 and 100 copies per cell. At 8 dpi, levels of viral DNA were increased in all organs except the spleen, where there was only about 1 copy of viral DNA per cell of viral DNA. Viral DNA replication between the 3- to 8-dpi period was most predominant in the kidney, where the highest level of viral DNA was at 8 dpi at between 100 and 1,000 copies per cell, a 10-fold increase over the level at 3-dpi. Virus was then cleared from all organs except the kidney, in which viral DNA persisted. At 30 dpi, there were between 1 and 10 copies of viral DNA per kidney cell. No viral DNA was present in the lungs after 8 dpi. These results were therefore the same as in previous experiments with i.p. infected mice (9, 16); that is, after i.p. infection, there was an initial acute phase of infection in which the lungs, liver, spleen, and kidneys were infected. Virus was cleared from all of these organs except the kidneys, in which polyomavirus persisted. Primary replication of virus was not limited to a single organ. Even though the lungs did become infected, as in i.n. inoculated mice, the levels of infection were much lower, and a persistent infection in the lung did not result after i.p. inoculation.

DISCUSSION

At least four distinct phases have been recognized after i.n. polyomavirus infection of mice. We refer to these phases as: (i) primary respiratory replication, (ii) secondary systemic replication, (iii) virus clearing, and (iv) persistent infection. Primary respiratory replication consists of the initial infection of the upper respiratory tract and associated tissues (Dubensky et al., in press). This phase culminated at about 6 dpi with a high level of viral DNA replication in the lungs. The secondary systemic phase of replication had then begun and was evidenced by infection of most of the visceral organs. This phase lasted until 10 to 12 dpi, at which time the clearing phase of infection was seen. By 22 dpi, polyomavirus DNA was cleared from all organs except the lungs and kidneys, in which a lower level of viral DNA persisted and slowly decreased over several months. This last phase is the persistent phase of infection. After i.n. infection, the levels of viral DNA persisting in the lungs were substantially higher than that seen in the kidneys. This persistent lung infection has not previously been observed.

The results from i.p. infected mice showed only three phases and were similiar to previous reports by others (16). We observed an initial systemic infection upon virus inoculation. This systemic infection closely resembled the secondary replication described above and involved the same organs, albeit to different levels of replication. This is followed by clearing of virus from all organs except the kidneys, in which a persistent infection results (11). Although polyomavirus replication in the lungs did occur, a persistent infection did not result there. This failure to persist in the lungs was somehow due to the route through

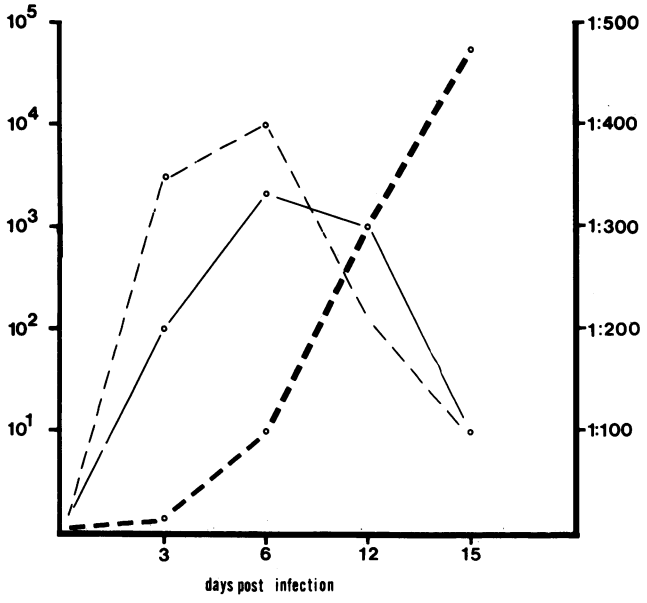

FIG. 5. Antibody and infectious virus titer in blood. Mice were bled at 3, 6, 12, and 15 dpi (i.n.). The unbroken line represents the virus titer per milliliter in heparinized whole blood, and the thin broken line represents the virus titer per milliliter in serum. The thick broken line represents the polyomavirus plaque-neutralizing antibody level.

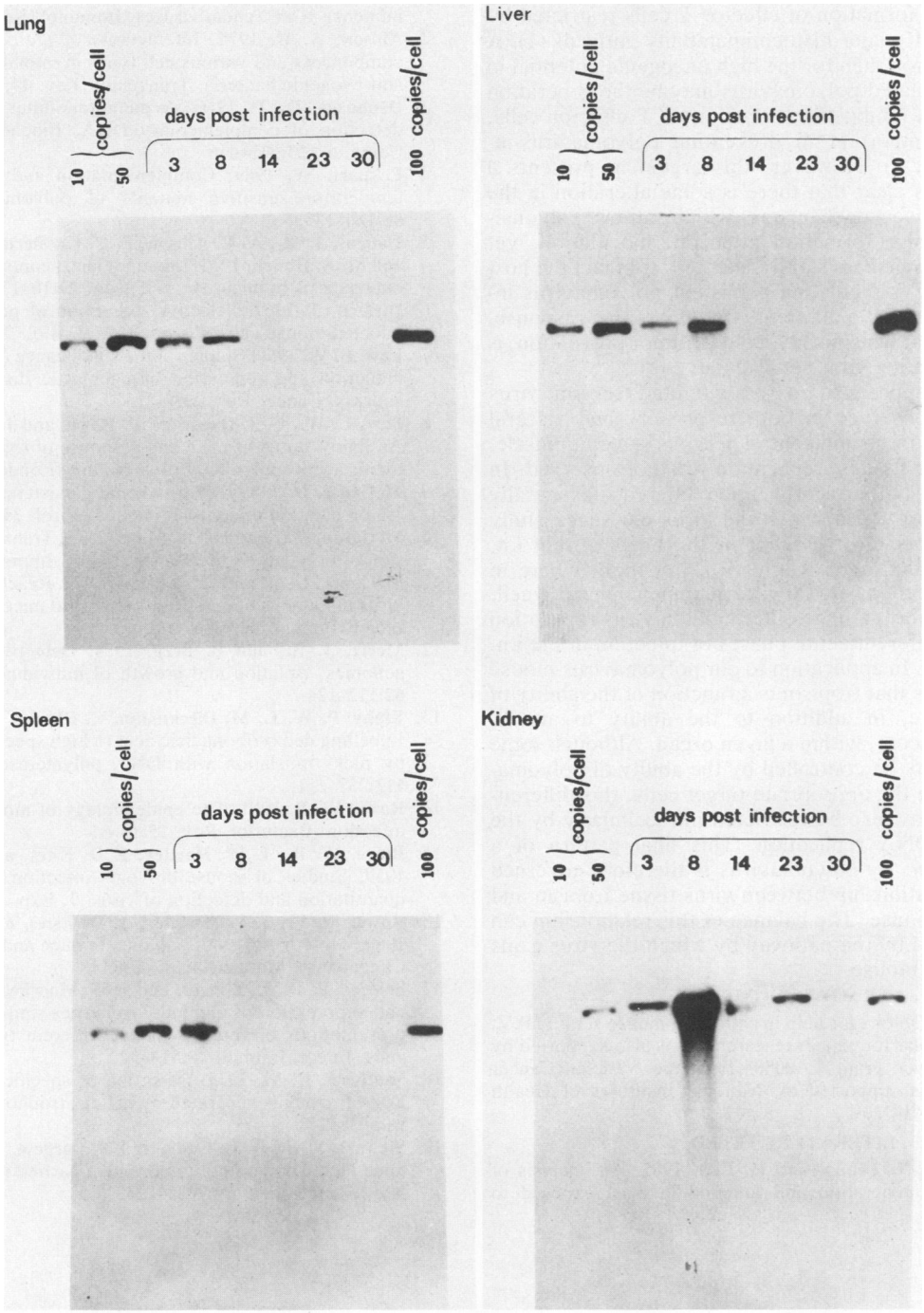

FIG. 6. Quantitation of viral DNA in i.p. infected mice. Tissue DNA (10 μg) at 3, 8, 14, 23, and 30 dpi were used. Copy number per cell reconstruction tracks were calculated as described in the text. All tracks were digested with *Bam*HI. Exposure times were 12 h for the kidney panel, 18 h for the lung and spleen panels, and 24 h for the liver panel.

which the virus arrived at the lungs. The virus initially encounters the respiratory epithelium in the lungs after i.n. instillation, whereas after i.p. inoculation, the virus gains access to the lungs via the blood. The level to which the virus replicates in the lungs is much higher after i.n. infection than after i.p. infection. Although we have yet to determine the reasons for the different levels of virus replication, two possibilities occur to us. It may be that different lung tissues are infected through these two routes and that virus replication is partially restricted via the i.p. route. Alternatively, nonspecific immune mechanisms (such as interferon) may restrict the delayed lung infection that occurs after i.p. inoculation. The fact that the i.p. lungs showed much lower levels of virus replication could also be related to inability to establish a persistent infection there.

This variance in host reactions to different routes of virus inoculation has been observed with both Sendai virus and herpes simplex virus infection of mice (1, 17). In the Sendai-mouse system, intravenous virus inoculation (but not i.n. inoculation) leads to the formation of suppressor T cells (T_s)

which inhibit the formation of effector T cells restricted by class I and class II major histocompatability antigens (1). A possible explanation then for the high oncogenic potential in mice of i.p. inoculated polyomavirus may be the generation of T_s cells which inhibit the formation of T effector cells, which may be important in preventing polyomavirus-induced tumors (2, 5). However, this argument presents a paradox: it seems clear that there is some alteration in the immunosurveillence mechanisms which allow polyomavirus-induced tumor formation after i.p. inoculation, yet fewer organs become persistently infected. If in fact the host immune response is regulating persistent polyomavirus infections, it may be that a different type of immune response, compared with that which is involved in tumor prevention, is involved in regulating viral persistence.

Polyomavirus appears to possess a limited tropism; virus replication was observed in both respiratory and visceral organs, but in the brain and central nervous system, muscle, and various other tissues, replication was not observed. In addition, the levels of virus replication differed substantially among infected organs in which the virus did successfully replicate. This was most evident in the lungs during i.n. infection and in the kidneys after i.p. infection, where in each case there were ca. 1,000 viral genome copies per cell. There was, therefore, a huge differential in virus replication during the two different acute phases of infection in the i.n. and the i.p. cases. In application to our polyomavirus-mouse system, it appears that tropism is a function of the ability of virus to replicate, in addition to the ability to attach, penetrate, and uncoat, within a given organ. Although some tissue tropism may be controlled by the ability of polyomavirus to penetrate the appropriate target cells, this differential expression may also be restricted intracellularly by the levels of viral DNA replication. This final pattern of a persistent infection by polyomavirus is therefore governed by a dynamic relationship between virus tissue tropism and host immune response. The balance of this relationship can clearly be affected by the pathway by which the virus gains entrance into the mouse.

ACKNOWLEDGMENTS

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