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A Determinant of Polyomavirus Virulence Enhances Virus Growth in Cells of Renal Origin

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We have identified a strain of polyomavirus, Py(L), which is unusual in causing acute morbidity and early death after inoculation of newborn mice. We determined that these animals died of kidney failure associated with extensive, virus-mediated destruction of renal tissue. Interestingly, the Py(L) strain infects baby mouse kidney cell cultures more efficiently than do other strains.

Characterization of the molecular determinants of viral virulence is an important step toward understanding the biology of virus infections, a major cause of morbidity and mortality in animals and humans. Several lines of evidence suggest that virulence is likely to be determined by multiple factors which make independent contributions to the capacity of a virus to cause illness in an infected host; attempts to identify these determinants have focused on both structural and nonstructural viral proteins. After reovirus infection, virulence is determined by the three outer capsid proteins, each of which plays a different role (5, 13). Attenuation of influenza viruses has been associated with alterations both in structural glycoproteins and in polypeptides important for viral RNA replication (6, 12). Other viruses for which virulence determinants have been examined include rhabdoviruses (3), other paramyxoviruses (17), herpesviruses (1, 14, 21), and retroviruses (2).

While natural infection by polyomavirus (Py) is thought to be silent (4), laboratory inoculation of newborn mice with large doses of Py may lead to a runting syndrome (9, 23) and a variety of tumors (4, 18, 19). In contrast to this widely observed but very limited spectrum of pathogenic effects, Main and Dawe noted in 1966 that a large proportion of mice inoculated with the LID strain of Py died or became moribund within 72 days after inoculation (11). We examined the virulence of two strains of wild-type Py, Py(P), our standard laboratory wild-type strain, and Py(L), in C3H/Bi/Da (C3H) mice by determining the survival of animals inoculated at 12 to 24 h after birth with different amounts of virus (Fig. 1) which had been titered by plaque assay on secondary mouse embryo cells (MEC) prepared from NIH general purpose mice. The Py(P) strain is the wild type of Py which has been used in our laboratory for all previous studies involving Py. It had been derived as a plaque isolate from MEC infected with the Pasadena large plaque strain of Py (24). Py(L) is a plaque isolate from MEC infected with the LID strain of Py, which was originally isolated from a mouse salivary gland tumor (11). Inoculation with Py(P) did not cause any significant morbidity or mortality within the first 40 days of life. Except for animals which developed Py-induced tumors many months after virus inoculation, these animals seemed to survive normally when compared with uninoculated controls (data not shown). The absence of any significant acute morbidity associated with wild-type Py infection in these animals during the newborn period is consistent with the findings of other investigators. The survival of animals inoculated with Py(L), however, contrasted sharply with that observed in animals inoculated with Py(P) (Fig. 1). Administration of as little as 7.5×10^3 PFU per animal led to ca. 50% mortality by 40 days after infection; inoculation of 7.5×10^5 PFU of Py(L), 10% the amount of Py(P) administered in the experiment shown in Fig. 1, led to 100% mortality within 12 days. Very few additional deaths occurred in any of these animals during a 60-day observation period after termination of the experiment shown in Fig. 1.

To evaluate the morbidity of Py infection and ascertain the cause of death in animals dying after inoculation with Py(L), we performed complete autopsies and a series of clinical studies on animals sacrificed on days 2, 4, 6, 8, 10, 12, and 14 after infection with either Py(L) or Py(P) (see Fig. 2 through 4). We examined animals inoculated with either 7.5×10^6 PFU of Py(P) or 2.5×10^5 PFU of Py(L). This dose of Py(P) represents 50 μl of undiluted virus stock and was the largest inoculum we could easily administer to newborns. We chose to examine animals inoculated with 2.5×10^5 PFU of Py(L) because of the extreme difficulty in obtaining adequate numbers of surviving animals for study at times after 6 days when mice were inoculated with higher doses of this strain (Fig. 1). A detailed description of the pathological findings in these animals will be presented elsewhere; however, a number of remarkable differences between animals inoculated with Py(P) and Py(L) were observed.

The frequency and extent of Py-associated lytic lesions were greater in animals inoculated with Py(L) than in animals inoculated with Py(P). Lesions extensive enough to explain the early death of animals inoculated with Py(L) were consistently found only in their central nervous systems and kidneys. In the central nervous systems of animals inoculated with Py(L), we found that hemorrhages surrounding small vessels with disrupted epithelium were present in many different locations. Although these hemorrhages may have arisen from Py-mediated damage to blood vessels, a possible etiology is hypertension secondary to extensive renal damage. Similar hemorrhages were not present in animals inoculated with Py(P). Mice with these hemorrhages also had extensive renal lesions; indeed, all mice which received Py(L) had renal parenchymal lesions which were easily detectable by day 4 after inoculation and were far advanced by day 10 after inoculation. These lesions were invariably associated with renal epithelium which contained

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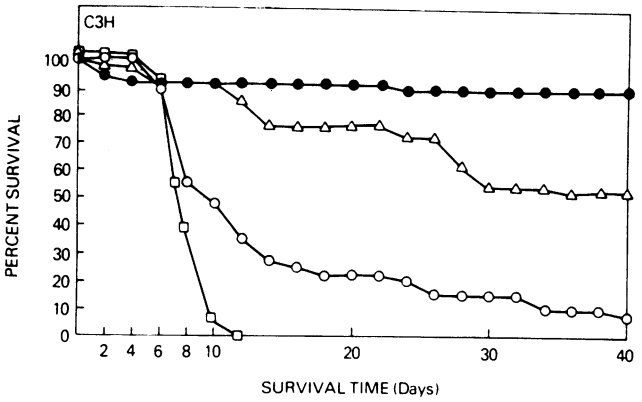

FIG. 1. Mortality of mice inoculated with Py. Newborn C3H/Bi/Da mice were inoculated with 7.5×10^6 PFU of Py(P) (●) or 7.5×10^5 (□), 2.5×10^5 (○), or 7.5×10^3 (△) PFU of Py(L) and examined every other day.

Py detectable by immunohistological examination (Fig. 2) and showed changes characteristic of lytic Py infection: ballooning degeneration, intranuclear inclusion bodies, and cellular disintegration (10). Typically, the cortical renal tubules were most extensively involved, although more distal tubular epithelia as well as glomerular epithelium was occasionally involved. Mice that received Py(P) developed minimal renal lesions by days 10 to 14 after inoculation; however, these were never extensive enough to suggest serious impairment of renal function.

Figure 2 shows histological sections of kidney tissue from animals sacrificed 8 days after inoculation with either Py(P) or Py(L). These sections have been incubated with rabbit anti-Py capsid protein antisera and then reacted with goat anti-rabbit immunoglobulin antisera to which horseradish peroxidase was covalently linked. Development of the horseradish peroxidase reaction with diaminobenzidine and H_2O_2 results in precipitation of brown granules that indicate foci of Py infection in these hematoxylin-stained sections. In the kidneys of Py(P)-infected animals (Fig. 2A), only a few small foci of lytically infected cells were visible. In the kidneys of Py(L)-infected animals (Fig. 2B), the outer cortical region contained large numbers of infected cells, predominantly of upper nephron epithelium. There was also a less conspicuous zone of positive reaction at the corticomedullary junction where medullary mesenchyme, as well as lower nephron epithelium, were lytically infected.

In experiments shown in Fig. 3, we found that the amount of Py present in the kidneys of the Py(L)-infected animals was much greater than that in the kidneys of animals that received 30 times more Py(P) and corresponded well to our detection of significantly more extensive pathological changes in the kidneys of animals inoculated with Py(L). These pathological findings suggested that the increased mortality of animals inoculated with Py(L) was associated with significant kidney damage in these animals. To further examine this possibility, we evaluated the kidney function of animals inoculated with either Py(P) or Py(L). Body weight was determined by weighing individual animals, and kidney weight was determined by weighing both kidneys after removal of perirenal fat and adherent adrenal tissue. The blood urea nitrogen levels in serum prepared from whole blood obtained by cardiac puncture were determined by an enzymatic method (20). The hematocrit of this blood is reported as the percentage of packed cells in a centrifuged specimen of heparinized whole blood.

Renal function, as measured by blood urea nitrogen, deteriorated as early as day 10 after inoculation with Py(L), although animals inoculated with 30 times more Py(P) showed no evidence of kidney malfunction (Fig. 4). This evaluation in blood urea nitrogen correlated with a decreased kidney weight and overall body weight in animals inoculated with

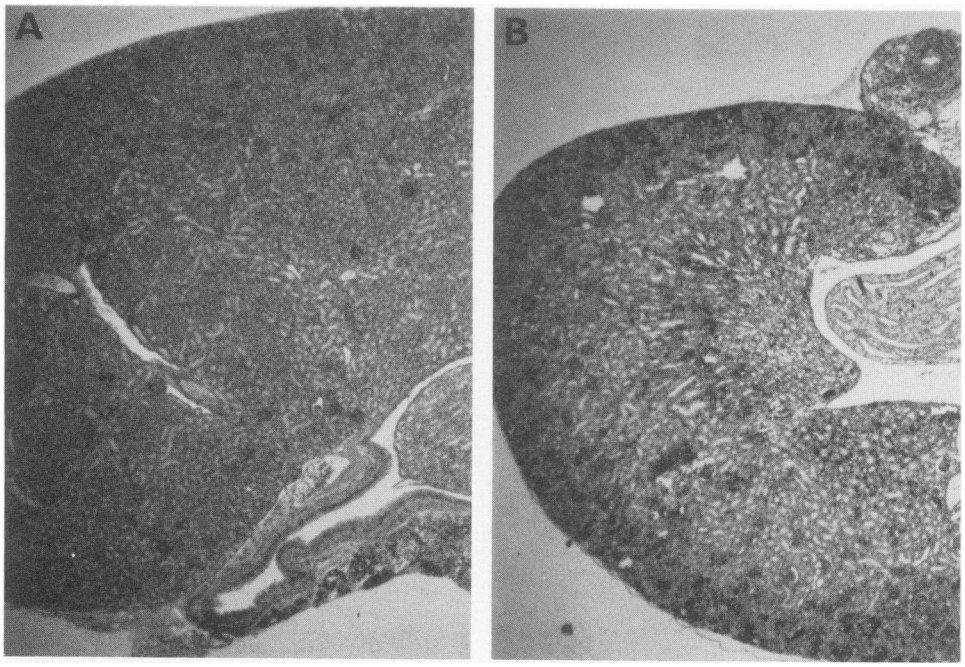

FIG. 2. Detection of Py in the kidneys of infected C3H/Bi/Da mice. Histological sections of kidneys from 8-day-old mice infected with either 7.5×10^6 PFU of Py(P) (A) or 2.5×10^5 PFU of Py(L) (B) were examined. The extent of Py infection is indicated by the dark, granular deposits resulting from a positive immunoperoxidase reaction, showing the presence of Py proteins.

Py(L) and occurred at approximately the same time at which the mortality of Py(L)-infected animals became pronounced (Fig. 1). We do not believe that this elevated blood urea nitrogen reflected morbidity in other organs, leading to general debilitation or dehydration, since the hematocrit of Py(L)-infected animals did not differ significantly from that observed in uninfected or Py(P)-inoculated animals (Fig. 4).

Because of the differences in Py-induced pathogenicity, we examined the kinetics of virus replication in C3H MEC and mouse 3T3 cells which were infected with 10 PFU of either Py(L) or Py(P) per cell. Despite repeated evaluations, we could detect no difference in the amount of Py(L) and Py(P) DNA synthesized in these cells at various times (12 to 96 h) after virus infection (data not shown). Similarly, both viruses grew to high titer (>5 × 10^8 PFU/ml) in MEC cultures and efficiently formed plaques on MEC and mouse 3T3 cells (data not shown). Since we observed a sharp contrast between Py(L) and Py(P) infection in the kidneys of infected animals (Fig. 2 and 3), we reasoned that differences in the efficiency of viral infection might be most easily detected in cells of kidney origin. The results of representative experiments examining three measures of Py infection of primary baby mouse kidney (BMK) cells are presented in Fig. 5.

Initially, we examined the amount of the Py major structural protein (VP_1) in C3H BMK cells infected with these viruses. We infected subconfluent monolayers of primary C3H BMK cells with 10 PFU of Py per cell. After a 1.5-h adsorption period at 37°C, the cells were washed three times with serum-free medium to remove nonadsorbed virus.

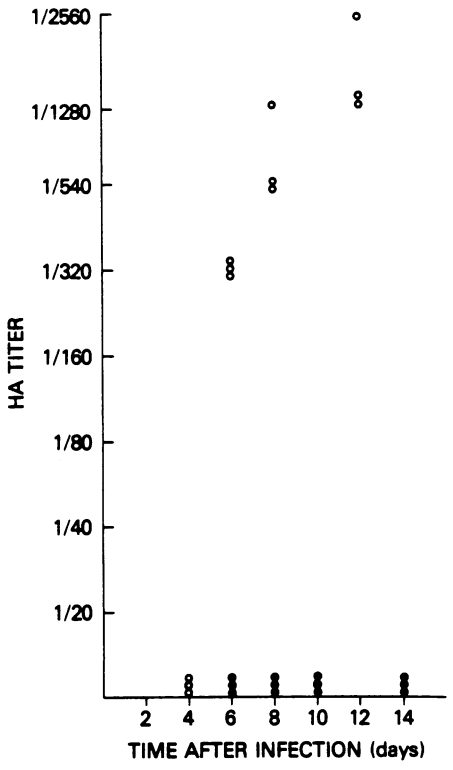

FIG. 3. Quantitation of Py in kidneys of infected C3H/Bi/Da mice. The amount of Py was estimated by determining the hemagglutination titer of 10% homogenates of renal tissue from individual animals of various ages after inoculation as newborns with either 7.5 × 10^6 PFU of Py(P) (●) or 2.5 × 10^5 PFU of Py(L) (○).

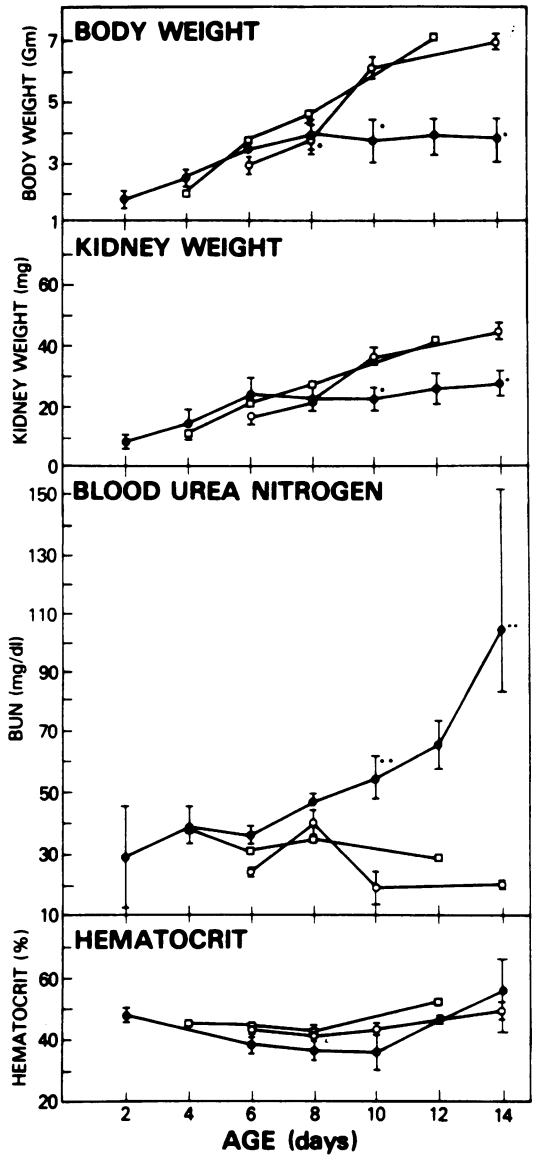

FIG. 4. Clinical evaluation of uninfected and Py-infected mice. Newborn C3H/Bi/Da mice were inoculated with 2.5 × 10^5 PFU of Py(L) (●), 7.5 × 10^6 PFU of Py(P) (○), or 50 μl of tissue culture media from uninfected mouse cells (□) and evaluated at 2, 4, 6, 8, 10, 12, and 14 days after birth. Each point on these graphs represents the average value obtained from evaluation of at least 3 animals; in most cases, 5 to 10 animals were examined. The bracketed values represent the range of values we obtained. The *P* value of the observed differences between the Py(L)- and Py(P)-inoculated animals at specific times after infection was determined and is indicated by * ($P < 0.01$) or ** ($P < 0.001$). The range of values observed for the control animals was <10% and was omitted for clarity.

Individual plates of identically handled cells were harvested at 12, 24, 48, and 72 h postinfection by the addition of 0.5 ml of lysis buffer (50 mM Tris-hydrochloride [pH 7.4], 0.5% Nonidet P-40, 5 mM $MgCl_2$, 25 μg of DNase per ml, 100 μg of aprotinin per ml, 25 μg of tolylsulfonyl phenylalanyl chloromethyl ketone) for 15 min at 23°C. The cellular extracts were adjusted to a final concentration of 2% sodium dodecyl sulfate (SDS)–5% β-mercaptoethanol and heated at 95°C for 10 min. Portions (50 μl) of the various extracts from

identical numbers of cells were subjected to electrophoresis on a 10% SDS-polyacrylamide gel and transferred electrophoretically to nitrocellulose (22). The resulting blots were reincubated with TNE buffer (20 mM Tris-hydrochloride [pH 7.5], 50 mM NaCl, 2.5 mM EDTA) containing 4% bovine serum albumin for 4 h and then incubated with a 1:50 dilution of goat antisera directed against Py VP_1 (from R. A. Consigli, Kansas State University, Manhattan) in the same buffer for 12 h. The blots were then extensively washed with TNE buffer, and the bound anti-immunoglobin G was detected by incubation with ^{125}I-labeled protein A (Amersham Corp.). The iodinated bands were localized by autoradiography. Bands corresponding to VP_1 were excised, and the radioactivity in these bands was quantitated by gamma scintillation spectroscopy (Fig. 5A). The presence of indistinguishable amounts of VP_1 in C3H BMK cell extracts at 12 h after infection with 10 PFU of either Py(L) or Py(P) per cell reflects the input virus and suggests that these viruses are taken up by C3H BMK cells with similar efficiencies; the finding of more VP_1 synthesis in C3H BMK cells after Py(L) infection than in Py(P)-infected cells indicates an enhanced efficiency of Py(L) infection.

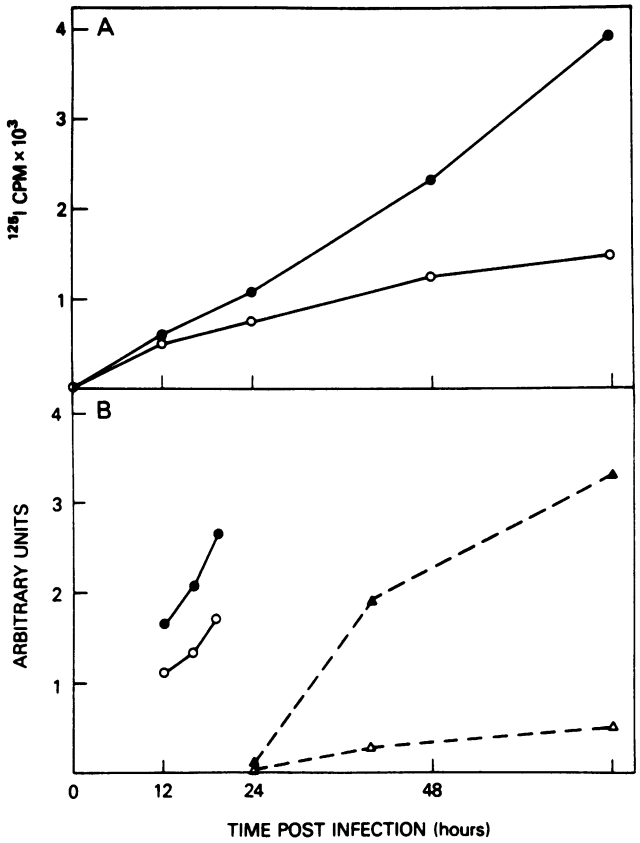

FIG. 5. Py(L) and Py(P) infection of BMK cells. (A) Equal amounts of cell extracts from C3H BMK cells infected with 10 PFU of either Py(L) (●) or Py(P) (○) per cell were electrophoresed on a 10% SDS-polyacrylamide gel. The separated proteins were electrophoretically transferred to nitrocellulose and allowed to react with monospecific goat anti-Py VP_1 antisera. The immune complexes were localized and quantitated as described in the text. (B) Equal amounts of [^{35}S]methionine-labeled cell extracts from C3H BMK cells infected with 10 PFU of either Py(L) (●) or Py(P) (○) per cell were immunoprecipitated with sera from rats bearing Py-induced tumors at the indicated times postinfection. After SDS-polyacrylamide gel electrophoresis of the immunoprecipitated Py T Ags and autoradiography, the extent of Py large-T Ag synthesis was estimated by densitometry tracing of the large-T Ag band. Py DNA synthesis was similarly determined by densitometry tracing of a ^{32}P-labeled band resulting from *Bam*HI cleavage of Py ^{32}P-labeled DNA isolated from equivalent numbers of C3H BMK cells infected with 10 PFU of either Py(L) (▲) or Py(P) (△).

To examine the kinetics of viral DNA synthesis, we examined $^{32}P_i$ incorporation into Py DNA isolated from C3H BMK cells infected with 10 PFU of either Py(P) or Py(L) per cell. At various times after infection, these cells were labeled for 1 h at 37°C with carrier-free $^{32}P_i$ (100 μCi/ml) in phosphate-free medium. Viral DNA was then isolated by differential salt precipitation (7) and subsequent cesium chloride-ethidium bromide equilibrium density centrifugation (15). After cleavage with *Bam*HI (Bethesda Research Laboratories), the viral DNA from equivalent numbers of cells was evaluated by electrophoresis in 1.0% agarose, and autoradiograms of these gels were analyzed by densitometry. The results of this experiment are shown in Fig. 5B and indicate that Py(L) replicated more efficiently than Py(P).

To further examine this enhanced capacity of Py(L) for virus replication in renal cells, we evaluated the synthesis of the Py large-tumor antigen (T Ag), the major protein product of the Py early region, in Py(L)- and Py(P)-infected C3H BMK cells at various times before the onset of detectable viral DNA replication. Cells were labeled at various times after infection for 3 h at 37°C in methionine-free medium containing 2% dialyzed fetal calf serum with [^{35}S]methionine (300 μCi/ml; specific activity, 1,300 Ci/mmol; Amersham). Extraction of the labeled proteins, immunoprecipitation of Py T Ags with sera from rats bearing Py-induced tumors, and SDS-polyacrylamide gel electrophoresis were carried out as previously described (8). Densitometry evaluations of autoradiograms of these polyacrylamide gels containing immunoprecipitated Py large-T Ag from identical numbers of cells were performed and analyzed on a Beckman DU-8 scanning spectrophotometer. More large-T Ag was detected in Py(L)-infected C3H BMK cells than in Py(P)-infected cells at these very early times (Fig. 5B), indicating that the efficient replication of Py(L) is associated with enhanced early production of Py(L)-encoded large-T Ag.

Although the pathological findings associated with any virus infection are the result of both host and viral factors, little is known of the molecular mechanisms by which such interactions are mediated. Infection of newborn C3H mice with the wild-type Py(L) causes extensive renal damage, significant morbidity, and early death. A disease with similar clinical and pathological characteristics has been recently ascribed to infection by a human Py, BK (16). Our finding that the destruction of renal tissue by Py(L) was associated with an enhanced ability of Py(L) to infect kidney cell cultures suggests strongly that the morbidity and mortality associated with Py(L) infection was determined in part by an interaction of viral sequences and tissue-specific host cellular factors which determined the efficiency of Py infection of renal tissue. Our observations that there are equivalent amounts of VP_1 in BMK cells immediately after infection with both Py(L) and Py(P) and that these viruses infect MEC and mouse 3T3 cells with indistinguishable efficiency suggest that the enhancement of Py(L) infection is not due to the inability of these mouse cells to take up Py(P). Rather, it seems more likely that other determinants of infectivity, such as viral gene expression and viral DNA replication, play important roles in determining the particular virus-host cell interaction we have observed. Indeed, whether one

considers that most wild-type Py strains, which do not kill newborn mice, are specifically attenuated or that Py(L) is particularly virulent, these viruses provide a unique opportunity to determine the molecular basis of pathogenic changes observed after papovavirus infection.

The pathogical effects of a virus infection, however, are determined not only by interactions between the virus and the individually infected host cells but also by the immunological response of the animals in which the infection occurs. Although the role of host defenses, including both humoral and cellularly mediated immune responses, has not been evaluated in these studies, we have found that mortality of inoculated newborn mice is dose dependent (Fig. 1) and that inoculation of immunologically competent weanling and adult C3H mice with virulent Py strains does not result in increased mortality when compared with uninoculated control animals (data not shown). These results suggest that the host immune response does play an important role in the development of the renal pathology we observed. Considering the relative simplicity of the Py genome and the ease with which it can be manipulated through recombinant DNA technology, it should be possible to decipher the regions of the viral genome contributing to the virulence of this particular viral strain.

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