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Characterization of Mouse Parvovirus Infection by In Situ Hybridization

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Infection of young adult BALB/cByJ mice with mouse parvovirus-1, a newly recognized, lymphocytotropic, nonpathogenic parvovirus, was examined by in situ hybridization. Virus appeared to enter through the small intestine and was disseminated to the liver and lymphoid tissues. Strand-specific probes detected virion DNA in a consistently larger number of cells than replicative forms of viral DNA and/or viral mRNA. The number of signal-positive cells in the intestinal mucosa, lymph nodes, spleen, and thymus increased through day 10 after oral inoculation but decreased after seroconversion. Positive cells were still detected, however, in peripheral lymphoid tissues of mice examined at 9 weeks postinoculation. The results underscore the need to assess potential effects of persistent mouse parvovirus-1 infection on immune function in mice.

Mouse parvovirus-1 (MPV-1) is an autonomous parvovirus of mice that was recently isolated from a mouse $CD8^+$ T-cell clone (22). The region of the MPV-1 genome encoding nonstructural proteins closely resembles that of minute virus of mice, but the region encoding capsid proteins differs significantly from minute virus of mice (2), which accounts for antigenic differences between these viruses (27). MPV-1 replicates productively in cloned T cells in vitro and inhibits their proliferative response to interleukin 2 or antigen but does not appear to inhibit the generation of cytotoxic T cells in mixed lymphocyte cultures (22). Initial in situ hybridization (ISH) studies, using infant euthymic mice and adult *scid* mice, indicated that MPV-1 infection is lymphocytotropic in infant and adult mice and demonstrated viral DNA in *scid* mouse tissues for at least 3 weeks after inoculation (27). Furthermore, virus was transmitted by contact for up to 6 weeks from euthymic mice inoculated as infants and for up to 4 weeks from mice inoculated as adults. Therefore, mice remained infectious after seroconversion, which occurs 7 to 10 days after inoculation of virus. These early findings suggested that MPV-1 causes persistent infection in adult immunocompetent mice. Current results confirm the development of persistent infection localized to lymphoid tissues and call for further assessment of immune function in infected mice.

MPV-1 was grown and titrated in a $CD8^+$ H-2L^d-reactive T-cell clone (L3) (22). Specific-pathogen-free, 4- to 6-week-old, female BALB/cByJ mice (The Jackson Laboratory, Bar Harbor, Maine) were inoculated orally with 300 50% tissue culture infective doses of virus. An initial group (Table 1) was used to examine infection with random-primed, ^{32}P-labeled DNA (double-stranded DNA [dsDNA]) probes (80 to 120 bp in length) prepared from a gel-purified *Eco*RI-*Xba*I fragment (nucleotides 383 to 4342) of MPV-1 (2). The brain, lung, heart, liver, pancreas, salivary glands, gastrointestinal tract (as Swiss rolls), thymus, spleen, lymph nodes (mesenteric, cervical, axillary, and popliteal), and urogenital tract from each mouse were fixed in periodate-lysine-paraformaldehyde (20), embedded in paraffin wax, and sectioned at 5 μm. Tissues were stained, hybridized, and evaluated as previously described (11).

None of the infected mice developed clinical signs, but the incidence of seroconversion to MPV-1, determined by immunofluorescence assay (27), was 100% by 10 days after inoculation. MPV-1-positive cells were first detected in the intestine, mesenteric lymph nodes, spleen, liver, and lung 3 days after inoculation (Fig. 1). Signal at this and subsequent stages of infection was typically over cell nuclei, the expected site of parvoviral replication. The number of positive cells in the intestine and lymphoid tissue increased through day 10 and then gradually decreased after serocoversion. Positive cells also were prominent in the liver during acute infection, whereas signal in other positive tissues—stomach, kidney, and lung—was sparse and transient. Some positive cells were detected in the small intestine and thymus through 3 weeks and in lymph nodes and spleen through 9 weeks. Tissue necrosis was not apparent at any stage of infection in adult mice used here or in prior studies of infected infant mice (27). This confirms previous findings that MPV-1 infection is essentially nonpathogenic and indicates that, if cytolytic infection occurs, the number of affected cells at any given time is too small to detect above normal levels of apoptosis.

Previous results showed that MPV-1 packages more than 99% minus-sense virion (2). Because plus-sense, strand-specific RNA probes (riboprobes) for parvoviruses detect virion DNA and replicative-form (RF) DNA and minus-sense riboprobes detect RF DNA and mRNA, they can be used together to distinguish sites of viral replication from sites of viral sequestration (1, 3). Therefore, tissues from a second group of MPV-1-infected mice (four to eight mice per time point) were hybridized with ^{35}S-labeled riboprobes, 100 to 600 bp in length, prepared from the same cloned fragment previously noted (2). Both riboprobes detected 1 pg of DNA on Southern blots, which is equivalent to approximately 4×10^5 copies of MPV-1. Groups of tissues for comparing detection by plus-sense and minus-sense riboprobes were always hybridized simultaneously. The onset, location, frequency, distribution, and duration of signal obtained with the plus-sense riboprobe resembled those obtained with the dsDNA probe. In contrast, labeling with the minus-sense riboprobe was sparse during acute infection and was not detected at a representative time

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TABLE 1. Incidence of MPV-1-positive tissues after oral inoculation of adult BALB/cByJ mice with virus, as determined by in situ hybridization of duodenum and lymphoid tissues with dsDNA probes for MPV-1

Tissue	No. of positive mice/total no. of mice at time postinoculation										
	Day							Week			
	2	3	4	5	6	7	10	2	3	6	9
Duodenum	0/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	3/4	0/8	0/10
Thymus	0/4	0/4	0/4	4/4	4/4	3/4	4/4	4/4	0/4	0/8	0/10
L nodes	0/4	2/4	3/3	4/4	4/4	4/4	4/4	4/4	3/4	5/8	10/10
Spleen	0/4	1/4	4/4	4/4	4/4	3/4	4/4	4/4	3/4	1/8	3/10
Total no. of mice	0/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	5/8	10/10

point (6 weeks postinoculation) during persistent infection (Fig. 2).

The number of cells positive for virion DNA-RF DNA consistently exceeded those positive for RF DNA-mRNA, during both acute and persistent infection. One potential explanation is that infected cells contain primarily sequestered rather than replicating virus. Alternatively, it could indicate that replication involves the production of comparatively large amounts of virion from small amounts of template that were undetected by the minus-sense riboprobe. In this context, Southern analysis of MPV-1 DNA extracted from tissues of adult mice has shown that single-stranded virion DNA predominates during acute infection (16). Discriminating between these alternatives will require more-sensitive techniques to detect replication, such as reverse transcriptase in situ PCR for detection of viral mRNA (19).

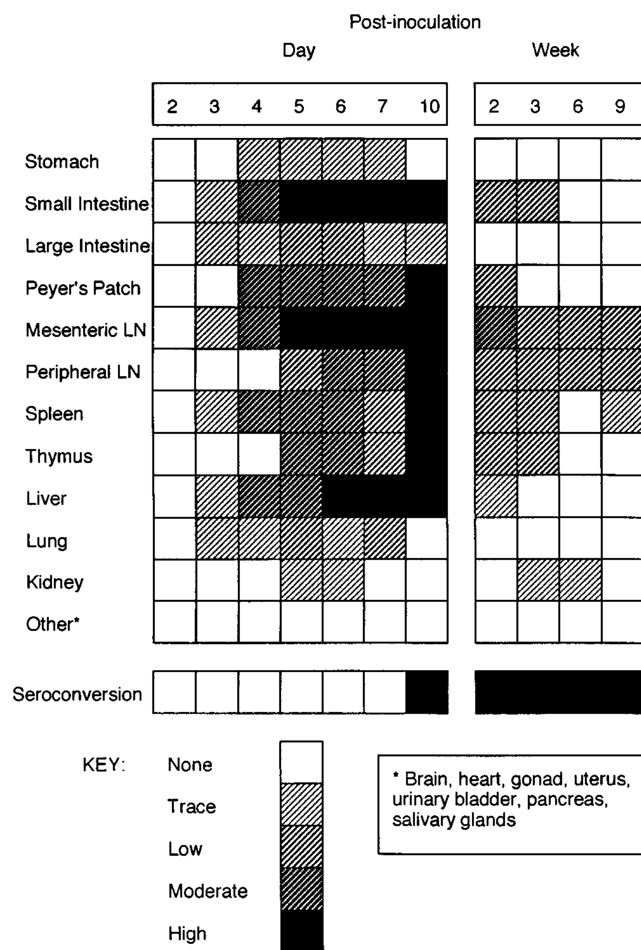

FIG. 1. Summary of ISH results with MPV-1 dsDNA probes on tissues from infected, adult BALB/cByJ mice. The prevalence of labeled cells in each tissue was estimated for representative low-power (10×) fields as negative, trace (one to 5 labeled cells), low (6 to 10 labeled cells), moderate (11 to 20 labeled cells), and high (>20 labeled cells). The survey of tissues was performed by in situ hybridization with a ^{32}P probe labeled by random priming. LN, lymph nodes.

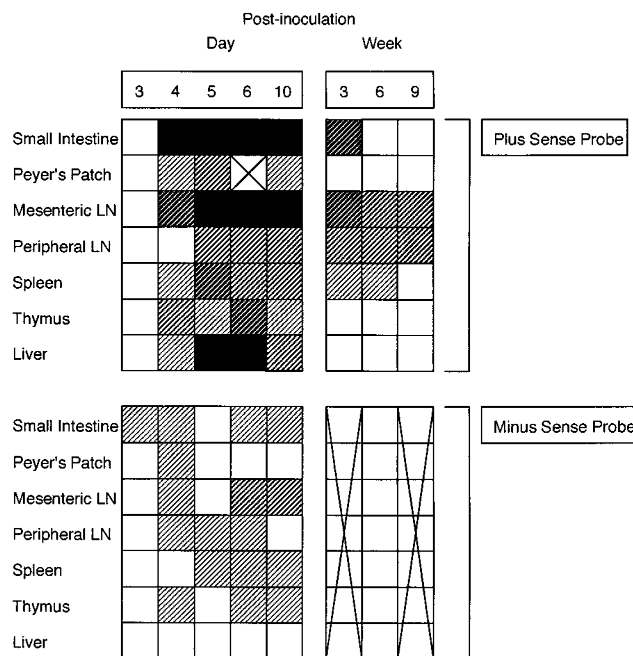

FIG. 2. Summary of ISH results with the single-stranded RNA probes for MPV-1. The minus-sense probe was used at five time points during acute stages of infection and at one representative time point (6 weeks) during persistent infection. (See Fig. 1 for key.) LN, lymph nodes.

Previous results suggested that the intestine was a site of viral entry, replication, and excretion (27). Several current findings support this hypothesis. After oral inoculation of virus, the small intestine was the first tissue in which positive cells were detected with both minus-sense and plus-sense riboprobes (day 3). Additionally, the mesenteric lymph nodes, which monitor intestinal lymphatic drainage, were the earliest site of signal among lymphoid tissues. Lastly, no signal was detected in mucosal epithelium of the stomach or upper alimentary tract. Viral entry and/or excretion through the respiratory or urinary tracts seem doubtful, since virus-positive cells were rare in both tissues. However, positive cells in the intestine appeared simultaneously among enterocytes and in mononuclear cells and capillary endothelium of underlying lamina propria. Furthermore, oral inoculation can result in some inhalation of virus. Therefore, the possibility that intestinal infection resulted from viremia after transient respiratory infection cannot be ruled out.

During the height of acute infection, mononuclear cells and capillary or lymphatic endothelium of the intestinal lamina propria were prominently labeled (Fig. 3). Labeling with both plus-sense and minus-sense riboprobes indicated that endothelium was a site of viral replication, a supposition consistent with the endotheliotropism of other rodent parvoviruses (5, 11,

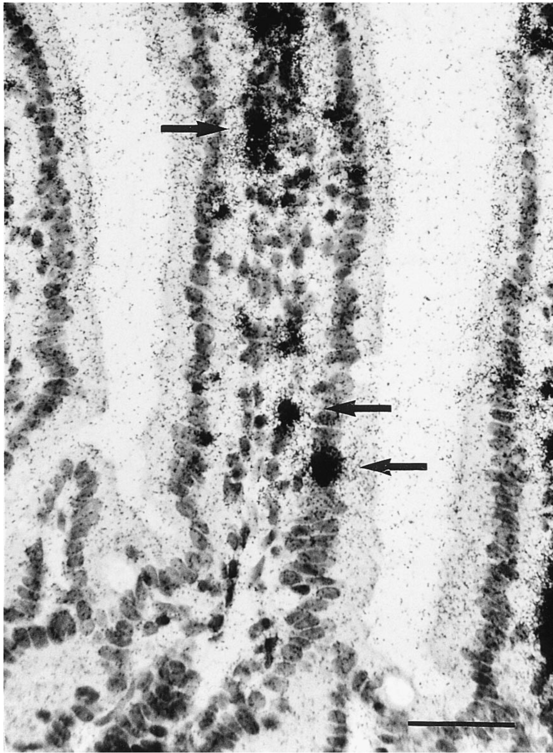

FIG. 3. Duodenal villus at day 6 postinoculation after hybridization with a plus-sense riboprobe to demonstrate nuclear signal for virion in enterocytes and in the lamina propria (arrows). Bar, 40 μm.

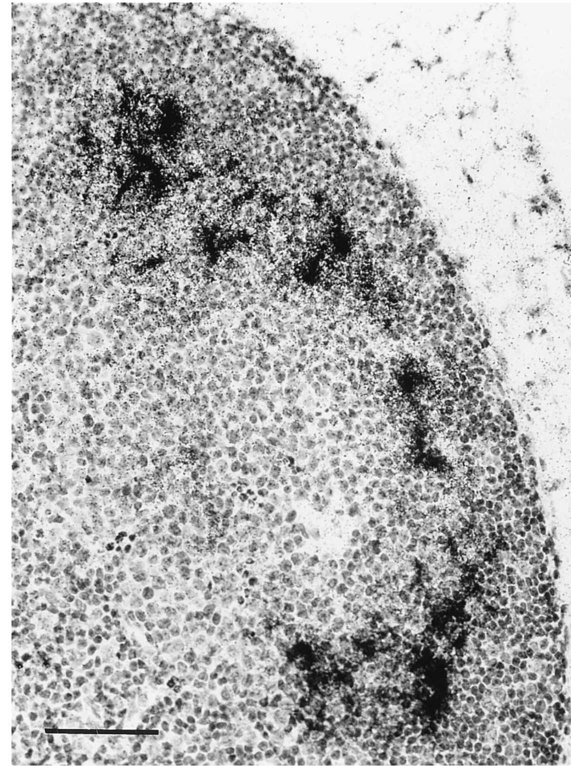

FIG. 5. Peripheral (cervical) lymph node labeled with a plus-sense riboprobe 10 days after inoculation of virus reveals early concentration of signal near a germinal center. Bar, 60 μm.

14, 17). Labeled enterocytes occurred occasionally among mitotically active cells of crypt epithelium, but they also were distributed randomly on villus walls (Fig. 3 and 4). Enteric parvoviruses of other species have a predilection for mitotically active crypt epithelium (6, 26, 29). Therefore, the random pattern of MPV-1-positive cells in the intestinal epithelium was unusual. This pattern could reflect a low level of viral replication in crypt cells that was not detected by ISH until the gradual accumulation of virus during cell differentiation and migration. Alternatively, viral replication could accelerate once a conducive stage of enterocyte differentiation had been attained during cell migration. Some positive cells in the enterocytic layer also may be infected intraepithelial lymphocytes (13). This possibility is consistent with the lymphocytotropism of MPV-1 and the presence of positive mononuclear cells in the lamina propria. Furthermore, MPV-1 is known to infect cloned γ/δ T cells in vitro (22). Double labeling of intestine for lymphocyte subsets (by immunohistochemistry) and MPV-1 (by ISH) should resolve this issue (23, 24).

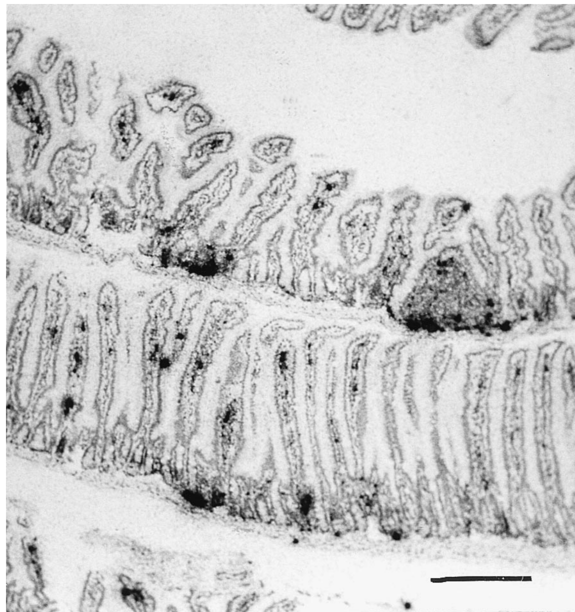

FIG. 4. Swiss roll of small intestine at day 6 postinoculation hybridized with a dsDNA probe to illustrate random distribution of positive cells among enterocytes as well as prominent signal in the lamina propria. A Peyer's patch also has conspicuous labeling. Identical results were obtained with the plus-sense riboprobe. Bar, 150 μm.

Although positive hepatocytes were observed occasionally, liver involvement consisted primarily of sinusoidal cells (Kupffer cells and endothelium) that were labeled only with the plus-sense probe. In addition, signal in many of these cells was less dense than in cells of intestine and lymphoid tissues at comparable stages of infection, suggesting that the liver was predominantly a site of viral sequestration resulting from phagocytic activity among littoral cells lining hepatic sinusoids.

Our results confirm that MPV-1 is lymphocytotropic in vivo. Positive cells occurred in the thymus (cortex and medulla) during the first 3 weeks of infection. Signal in spleen and lymph nodes was distributed randomly during the first week of infection and then localized in the vicinity of germinal centers and lymphoid follicles through 9 weeks (Fig. 5 and 6). Although bone marrow was not examined, only an occasional positive cell was found in splenic red pulp, suggesting that tropism for hematopoietic tissue is not a major feature of MPV-1 infec-

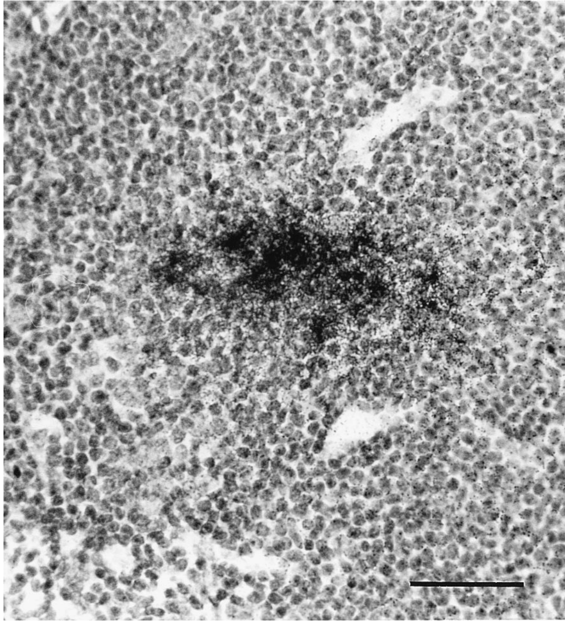

FIG. 6. Germinal center of a mesenteric lymph node labeled with a plus-sense riboprobe 9 weeks after inoculation. Bar, 40 μm.

tion. Signal in all lymphoid tissues was detected with both riboprobes during acute infection but was always more prominent with the plus-sense probe. During persistent infection, hybridization was detected only with the plus-sense probe.

The association of virus with lymphoid follicles and germinal centers is similar to patterns of lymphoid tissue infection found in parvoviral infections of other species (1, 6, 26, 29). Although MPV-1 infects cloned $CD4^+$, $CD8^+$, and γ/δ T cells in vitro, we have not yet determined if it has a predilection for specific subsets of lymphoid cells in vivo, as has been suggested for Aleutian disease virus of mink (25). Nevertheless, persistent lymphocytotropic infection in adult mice by MPV-1 adds to concerns about potential adverse influences of murine parvoviral infections on immune function. In this context, MPV-1 was shown to inhibit the proliferation of cloned T cells in vitro (22), and ongoing studies indicate that MPV-1 perturbs immune responses of BALB/c mice to a protein antigen (ovalbumin) and a transplantable tumor (sarcoma 180) (21). MPV-1 appears to differ in this respect from the immunosuppressive variant of minute virus of mice, which is not thought to alter immune responses of adult mice (4, 8, 18).

The onset of host immunity, measured by seroconversion, reduced but did not eliminate infection. A similar pattern has been observed in parvovirus infection of newborn rats wherein infectious virus can be recovered from immune rats even after ISH signal is sparse or no longer detected (9, 11). Localized viral persistence could result from cytolytic cell-to-cell infection fueled by mitotic activity to promote repair, as we have suggested previously (14, 15). In such a scenario, host immunity would prevent the dissemination of infection (that is, viremia) but would be less effective against localized infection. Alternatively, virus could remain sequestered (or replicating at low levels) in infected cells until mitotic activity (e.g., responses of lymphoid cells to stimulation by heterologous antigens) enhanced replication.

The relative importance of humoral versus cellular immunity in host defenses against rodent parvovirus infection has not been resolved. Passive immunization with immune serum prior to or coincident with inoculation of virus protects rats against lethal and persistent parvovirus infection but is not fully effective in eliminating preexisting infection (9, 10, 12). Comparable studies of parvovirus infection in mice have not been reported. A role for cell-mediated immunity in host defenses against parvoviruses is implied from mononuclear cell infiltrations observed in parvovirus infections of rats (11, 14) and mink (23), but functional evidence for cell-mediated immunity has not been reported. Mononuclear cell responses were, however, not observed during MPV-1 infection. While this may indicate that cellular immunity plays a relatively minor role, it also could indicate that MPV-1 suppresses or eliminates T cells that would nominally respond to infection. Preferential depletion of such T cells also may facilitate persistent infection, by compromising antiviral immune surveillance. We note, in this regard, that MPV-1 infects and lyses $CD8^+$ T cells in vitro (22) and that such cells are often important for host defenses against viral infections (7).

Current ISH results support the concept of persistent infection but would be strengthened by recovery of infectious virus from persistently infected mice. L3 cells, the only cell line known to support MPV-1 replication, are not ideal for viral bioassays because triturated tissues or explant culture lysates are toxic for them. The identification of other susceptible cell lines is, therefore, a priority for future MPV-1 research.

Apart from clarifying the impact of parvoviruses on laboratory mice, characterization of MPV-1 infection should contribute to general understanding of persistent parvoviral infection, including persistence of B19 virus, a human parvovirus associated with a variety of disorders including hydrops fetalis and aplastic anemia (28).

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