Persistent Rat Virus Infection in Smooth Muscle of Euthymic and Athymic Rats

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Rat virus (RV) infection can cause disease or disrupt responses that rely on cell proliferation. Therefore, persistent infection has the potential to amplify RV interference with research. As a step toward determining underlying mechanisms of persistence, we compared acute and persistent RV infections in infant euthymic and athymic rats inoculated oronasally with the University of Massachusetts strain of RV. Rats were assessed by virus isolation, in situ hybridization, and serology. Selected tissues also were analyzed by Southern blotting or immunohistochemistry. Virus was widely disseminated during acute infection in rats of both phenotypes, whereas vascular smooth muscle cells (SMC) were the primary targets during persistent infection. The prevalence of virus-positive cells remained moderate to high in athymic rats through 8 weeks but decreased in euthymic rats by 2 weeks, coincident with seroconversion and perivascular infiltration of mononuclear cells. Virus-positive pneumocytes and renal tubular epithelial cells also were detected through 8 weeks, implying that kidney and lung excrete virus during persistent infection. Viral mRNA was detected in SMC of both phenotypes through 8 weeks, indicating that persistent infection includes virus replication. However, only half of the SMC containing viral mRNA at 4 weeks stained for proliferating cell nuclear antigen, a protein expressed in cycling cells. The results demonstrate that vasculotropism is a significant feature of persistent infection, that virus replication continues during persistent infection, and that host immunity reduces, but does not eliminate, infection.

Materials and Methods

Virus and virus isolation. RV-UMass was obtained from Arthur Like, University of Massachusetts Medical School, Worcester. Virus stocks were prepared and quantified in NRK cells as previously described (26).

Rats. Pregnant Rattus rats, heterozygous at the mnu locus (mnu+/−), which had been mated with athymic (nu/nu) males, were obtained from the Animal Genetics and Production Branch, National Cancer Institute, Bethesda, Md. Litters consisted of approximately equal numbers of mnu+ (euthymic) and mnu/mnu...
Serum were stored individually at –70 °C to detect infectious virus. Blood samples were collected by cardiac puncture, and pieces of lung, kidney, and spleen collected at 2, 4, and 8 weeks were explanted as 3-mm fragments. Seven to nine fragments of each tissue were cultured, by tissue, (pH 8.0), 10 mM EDTA, and 250 μg of proteinase K per ml. Suspensions were centrifuged at 10,000 x g for 10 min prior to freezing with fresh Teflon chambers with stainless steel balls using a Dismembrana- tion, and dried. Samples were resuspended in Tris-EDTA containing 100 μg of DNA. Blots were washed twice for 30 min in 2× SSC–0.2% SDS and exposed to Kodak X-Omat AR film (Eastman Kodak, Rochester, N.Y.).

Molecular probes. Randomly primed 32P-labeled DNA probes were prepared using a commercial kit (New England Biolabs, Beverly, Mass.) and purified RNA-UMass DNA (nucleotides 1086 to 4300) as a template. The same template was used to prepare biotinylated randomly primed DNA probes to detect virus by immunoperoxidase staining augmented by tyramine-based amplification (catalyzed reporter deposition [CARD] probe) (NEN Life Science Products, Boston, Mass.).

Strong-specific 32P-labeled riboprobes (Promega, Madison, Wis.) were used to differentiate cells containing viral mRNA, an indicator of viral replication, from cells containing virion and RF DNA. RV-UMass DNA from nucleotides 2655 to 4277 was cloned into Bluescript KS vector (Stratagene, La Jolla, Calif.). RNA probes, detecting either plus-sense or minus-sense virus strand, were transcribed in equivalent amounts using the T7 promoter. The threshold for detection of viral mRNA in PLP-fixed cells with the plus-sense probe was established previously as 2.3 x 10⁹ copies (Boll-Goodrich et al., submitted).

ISH. Hybridization of tissue sections with randomly primed or strand-specific radiolabeled probes was performed and assessed as previously described (24). Tissue sections were hybridized with the same probe batch and same exposure times to minimize animal-to-animal and tissue-to-tissue variation in autoradiography. Assessment of radiolabeled hybridizations by light microscopy was based on a semiquantitative scale. Signal prevalence was defined as high (greater than 100 positive per section), moderate (50 to 100 positive per section), low (5 to 50 positive cells per section), trace (1 to 5 positive cells per section), or negative. A cell was scored as positive if the overlying grain count was at least 8 grains (twice background).

ISH preparation for the CARD probe was identical to that for the radiolabeled probe. The probe was denatured at 90°C for 4 min and cooled, and 2 ng was added to 50 μl of hybridization mix containing 60% formamide, 10% dextran sulfate, 2× SSC, and 50 μg of salmon sperm DNA per ml. Slides were covered, immersed in mineral oil, and incubated for 48 h at 42°C. Mineral oil was removed with three washes in chloroform, and coverslips were removed during two 5× SSC washes. Tissue sections were then washed twice in 60% formamide–2× SSC at 42°C, twice with 2× SSC at 42°C, and once at room temperature with 2× PBS. Staining was performed on 2× PBS at 60°C. Detection reactions were performed according to the manufacturer’s protocols (Renaissance, TSA-Indirect; NEN Life Science Products). Streptavidin-HRP was used at a dilution of 1:100 for both reactions, and tissue was biotinylated and dyed with biotin-tyramide for 10 min and 4,4-diaminobenzidine for 15 min. Tissue sections were counterstained with hematoxylin.

Immunohistochemistry for PCNA. Paraffin sections were dehydrated and hydrated. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide. Tissue was incubated for 45 min at 37°C with a mouse monoclonal antibody against proliferating-cell nuclear antigen (PCNA) linked to HRP by a flexible polymer backbone which facilitates attachment of numerous HRP molecules (Dako Corporation, Carpinteria, Calif.). After being washed in PBS, sections were exposed to 100 μg of 1-β-D-arabinofuranosylcytosine (ara-C) and 500 μg of mitomycin C per ml of PBS. Sections were then washed twice with PBS containing 0.1% Tween 20 and blocked with 5% horse serum. Sections were exposed to 4,4-diaminobenzidine (DAB) for 20 min. Sections were then washed, dehydrated, and mounted with Permount (National Diagnostics, Atlanta, Ga.).

Sections were first labeled for PCNA using the indirect immunoperoxidase reaction augmented by tyramine-based amplification (cardyem-35S-labeled, minus-sense RNA probe contain- ing 5 x 10⁵ cpm/μl of hybridization solution. Slides were washed as previously described and coated with autoradiographic emulsion (24). Emulsion was developed after a 27- to 30-h exposure to labeled tissue sections. One hundred thirteen cells containing viral mRNA in tissues of persistently infected rats were examined for coexpression of PCNA. Cells were scored as having strong, weak, or no PCNA staining.

Immunohistochemistry for RV NS and VP2. Immunofluorescence for the RV capsid protein (VP2) and the nonstructural protein (NS) was prepared during a prior study of RV infection (Boll-Goodrich et al., submitted). Selected tissues were stained by the avidin-biotin complex immunoperoxidase method (30).

RESULTS

Clinical signs and gross lesions. No clinical signs or gross lesions were found with either phenotype.

Virus isolation. Explant culture amplified infectious RV in tissue samples, so it is highly sensitive for detecting small quantities of infectious virus encountered during persistent RV infection (45). Lung, spleen, and kidney were assayed by explant culture at weeks 2, 4, and 8. All rats of both phenotypes had infectious virus through week 4, and all but one (a euthy-
mic rat) were virus positive at week 8 (Table 1). Twenty-seven of 29 tissues (93%) from euthymic rats tested through week 4 yielded infectious virus, but the prevalence decreased to 7 of 18 tissues (39%) by week 8. All tissue samples from athymic rats were positive through week 8.

**ISH with randomly primed probes.** A randomly primed, $^{35}$P-labeled DNA probe which detects a segment of the RV genome encoding the NS and VP genes was used to estimate the distribution and frequency of infected tissues and cells. It also served to detect virus in tissues that were not conducive to explant culture. A biotinylated CARD probe (28) was used to confirm infected cell types.

The radiolabeled, randomly primed probe revealed that euthymic and athymic rats developed widespread infection during the first 10 days after inoculation, consistent with previous results (24). Viral DNA was detected in thoracic and abdominal viscera by day 4, and the prevalence in positive tissues, including lymph nodes and spleen, was 100% on day 6 throughout week 2 (Table 2). Infected tissues contained few necrotic cells, in contrast to the severe necrosis which typifies acute infection in rats inoculated at 2 days of age. The frequency of virus-positive tissues declined by week 8 and to a greater extent among euthymic rats than athymic rats (Table 2).

The frequency of virus-positive cells in infected tissues was high in euthymic and athymic rats through day 10 (Table 3). It decreased progressively in both phenotypes from week 2 onward, but the decline was more pronounced in euthymic rats. By week 8, tissues of euthymic rats still contained positive cells, but in small numbers. All tissues examined in athymic rats at week 8 had positive cells at low-to-moderate prevalence.

Blood vessels were common sites of acute and persistent infection in both phenotypes. The aorta and pulmonary, mesenteric, renal, and gonadal arteries, in addition to smaller arteries and arterioles within lung, liver, kidney, and other tissues, were involved. During acute infection, signal was prominent among endothelial cells; however, it also was found in SMC within vessel walls. During persistent infection, the number of positive endothelial cells decreased, leaving SMC as the primary targets. This finding was confirmed using a biotinylated probe to examine tissues from two rats of each genotype at weeks 4 and 8. Infected SMC occurred singly or in groups and were distributed randomly or adjacent to the intima (Fig. 1A). Most SMC in infected vessels were histologically normal; however, some had swollen, vacuolated, angular, or pyknotic nuclei consistent with cell injury or impending death. Intramural hemorrhage and inflammation was not detected, however, in infected vessels. Virus-positive SMC also were found in the muscle tunics of the small intestine, especially in athymic rats. Lung was a site of persistent infection in rats of both phenotypes, and pneumocytes were the most commonly affected cells (Fig. 1B [left]). Respiratory epithelium also was infected in athymic rats. Renal tubular epithelium was a common site of acute infection, and positive cells were seen occasionally in rats of both phenotypes during persistent infection (Fig. 1B [right]). A few positive biliary epithelial cells and hepatocytes were found in the livers of persistently infected athymic rats.

**ISH with strand-specific riboprobes.** $^{35}$S-labeled, strand-specific riboprobes were used to detect the frequency and distribution of cells containing RV DNA and RV mRNA. The plus-sense probe was used to detect virion and replicative-form DNA, and the minus-sense probe was used to detect viral mRNA, indicative of active or recently completed replication (8). Tissues from two rats of each phenotype were examined at days 6, 8, and 10 and at week 2, and tissues from five to six rats of each phenotype were examined at weeks 4 and 8. Hybridization with the plus-sense riboprobe detected a slightly lower prevalence of positive cells compared to results using the randomly primed probes, indicating some reduction in sensitivity (Tables 4 and 5). This reduction was attributed largely to the lower energy and extent of labeling of the single-stranded $^{35}$S

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**TABLE 1. Prevalence of RV-UMass in lung, kidney, and spleen as determined by explant culture**

<table>
<thead>
<tr>
<th>Week</th>
<th>Phenotype</th>
<th>Lung-positive tissues</th>
<th>Kidney-positive tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>Euthymic</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>Athymic</td>
<td>4/4</td>
<td>4/4</td>
</tr>
<tr>
<td>4</td>
<td>Euthymic</td>
<td>5/5</td>
<td>6/6</td>
</tr>
<tr>
<td></td>
<td>Athymic</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>8</td>
<td>Euthymic</td>
<td>0/6</td>
<td>4/6</td>
</tr>
<tr>
<td></td>
<td>Athymic</td>
<td>5/5</td>
<td>5/5</td>
</tr>
</tbody>
</table>

*One sample per tissue was tested. Results are expressed as numbers of positive tissues (rats)/numbers of tissues (rats) examined.

**TABLE 2. Tissues containing RV detected by ISH using a randomly primed $^{35}$P-labeled probe**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Euthymic rats at week</th>
<th>Athymic rats at week</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2  4  6  8</td>
<td>2  4  8</td>
</tr>
<tr>
<td>Heart</td>
<td>4/4 4/6 1/6</td>
<td>4/4 5/5 3/5</td>
</tr>
<tr>
<td>Lung</td>
<td>4/4 6/6 4/4</td>
<td>4/4 5/5 5/5</td>
</tr>
<tr>
<td>Liver</td>
<td>4/4 6/6 0/6</td>
<td>4/4 5/5 5/5</td>
</tr>
<tr>
<td>Kidney</td>
<td>4/4 6/6 5/6</td>
<td>4/4 5/5 5/5</td>
</tr>
<tr>
<td>Spleen</td>
<td>4/4 5/5 2/6</td>
<td>4/4 5/5 5/5</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>4/4 5/5 6/6</td>
<td>4/4 5/5 5/5</td>
</tr>
<tr>
<td>Small intestine</td>
<td>4/4 5/6 2/6</td>
<td>4/4 5/5 5/5</td>
</tr>
<tr>
<td>Great vessels</td>
<td>4/4 6/6 5/6</td>
<td>4/4 4/4 5/5</td>
</tr>
<tr>
<td>Mesenteric vessels</td>
<td>4/4 6/6 2/4</td>
<td>4/4 5/5 5/5</td>
</tr>
<tr>
<td>Gonadal vessels</td>
<td>ND 3/3 3/3</td>
<td>ND 3/3 2/2</td>
</tr>
</tbody>
</table>

*Results are expressed as numbers of positive rats/numbers of rats examined. ND, not determined.

**TABLE 3. Mean prevalence of RV-positive cells detected by ISH using a randomly primed $^{32}$P-labeled probe**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Euthymic rats</th>
<th>Athymic rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 10 Week</td>
<td>Day 10 Week</td>
</tr>
<tr>
<td>Heart</td>
<td>+++ +/+ -/+</td>
<td>+++ +/+ -/+</td>
</tr>
<tr>
<td>Lung</td>
<td>+++ +++ +/+</td>
<td>+++ +++ +/+</td>
</tr>
<tr>
<td>Liver</td>
<td>+++ +++ +/+</td>
<td>+++ +++ +/+</td>
</tr>
<tr>
<td>Kidney</td>
<td>+++ +++ +/+</td>
<td>+++ +++ +/+</td>
</tr>
<tr>
<td>Spleen</td>
<td>+/+ +/+ +/+</td>
<td>+/+ +/+ +/+</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>+++ +++ +/+</td>
<td>+++ +++ +/+</td>
</tr>
<tr>
<td>Small intestine</td>
<td>+++ + +/+</td>
<td>+++ + +/+</td>
</tr>
<tr>
<td>Great vessels</td>
<td>+++ + +/+</td>
<td>+++ + +/+</td>
</tr>
<tr>
<td>Mesenteric vessels</td>
<td>+++ + +/+</td>
<td>+++ + +/+</td>
</tr>
<tr>
<td>Gonadal vessels</td>
<td>+++ + +/+</td>
<td>+++ + +/+</td>
</tr>
</tbody>
</table>

*Signal prevalence was defined as high (++) (>100 positive cells per tissue section), moderate (++) (50 to 100 cells positive per tissue section), low (+) (5 to 50 positive cells per tissue section), trace (+/-) (1 to 5 positive cells per tissue section), or negative (-). A cell was scored as positive if the overall grain count was at least 8 (twice background). The results are expressed as mean scores for the corresponding tissues from three to six rats per time point. ND, not determined.
riboprobe than was observed with the double-stranded, randomly primed $^{32}$P probe. The distribution of virus-positive cells was identical, however, to that detected by the randomly primed probes.

The prevalence of tissues and cells positive for viral DNA was consistently higher in rats of both phenotypes than that for viral mRNA. However, the prevalence of tissues and cells positive for viral mRNA during persistent infection was higher in athymic rats than in euthymic rats, except with peripheral (mesenteric) lymph nodes. In athymic rats, mesenteric lymph nodes contained ample RV DNA, especially in germinal centers, but no mRNA was detected. Nevertheless, viral mRNA

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Euthymic rats</th>
<th>Athymic rats</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Week 4</td>
<td>Week 8</td>
</tr>
<tr>
<td>Heart</td>
<td>3/6</td>
<td>1/6</td>
</tr>
<tr>
<td>Lung</td>
<td>6/6</td>
<td>4/6</td>
</tr>
<tr>
<td>Liver</td>
<td>5/5</td>
<td>2/6</td>
</tr>
<tr>
<td>Kidney</td>
<td>6/6</td>
<td>4/6</td>
</tr>
<tr>
<td>Spleen</td>
<td>0/6</td>
<td>0/6</td>
</tr>
<tr>
<td>Lymph nodes</td>
<td>5/5</td>
<td>5/6</td>
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<td>0/5</td>
</tr>
<tr>
<td>Mesentric vessels</td>
<td>2/5</td>
<td>0/5</td>
</tr>
<tr>
<td>Gonadal vessels</td>
<td>3/3</td>
<td>3/3</td>
</tr>
</tbody>
</table>

* Results are expressed as numbers of positive rats/numbers of rats examined.
positive cells were scored as negative for PCNA (Fig. 1D) and strong (18%). Therefore, approximately half of the mRNA-positive cells were scored for PCNA staining (strong, weak, or negative) by two independent observers using light microscopy. The mean counts were negative (48%), weak (34%), and strong (18%). Therefore, approximately half of the mRNA-positive cells were scored as negative for PCNA (Fig. 1D).

Colabeling for and PCNA and RV mRNA. The replication requirements of autonomous rodent parvoviruses, including utilization of host cell DNA polymerase, imply that RV mRNA should be detected primarily or solely in cycling cells. To test this expectation, tissues were stained for PCNA by immunohistochemistry followed by ISH for RV mRNA. PCNA is a highly stable protein which is first synthesized during late G1, attains peak concentrations during S phase of the cell cycle, and is not expressed during G0 (52). Staining for PCNA alone was performed in preliminary experiments to determine whether infected rats had more PCNA-positive cells than uninfected, age-matched control rats. One thousand to 1,500 hepatocytes were counted in each of four rats (one infected and one uninfected of each phenotype) 4 weeks after inoculation. The fractions of PCNA-positive cells were approximately equal in all livers: 54 and 59% in infected rats and 62 and 63% in uninfected rats.

Mesenteric and gonadal vessels and small intestine from two infected athymic rats at 4 weeks after inoculation were colabeled for PCNA and viral mRNA. A total of 113 mRNA-positive cells were scored for PCNA staining (strong, weak, or negative) by two independent observers using light microscopy. The mean counts were negative (48%), weak (34%), and strong (18%). Therefore, approximately half of the mRNA-positive cells were scored as negative for PCNA (Fig. 1D).

Southern analysis. DNA was prepared from athymic and euthymic rat tissues (spleen, kidney, liver, and lung) to enrich for small-molecular-weight DNA. The band pattern observed during acute infection (day 8) was similar to that established for RV-UMass replication in synchronized tissue culture cells (Ball-Goodrich et al., submitted). It included minus-sense (vi- rion), single-strand DNA which migrates at approximately 2.5 kb and two double-stranded, replicative forms which are approximately 5 kb (monomer) and 10 kb (dimer) (Ball-Goodrich et al., submitted). All three forms also were identified in athymic and euthymic tissues at weeks 4 and 8. However, signal intensity varied among the different tissues at these time points. In athymic and euthymic rats, the bands appeared to be stronger in euthymic tissues, especially in the liver, than in athymic tissues.

Antiviral immunity. The decrease in virus-positive cells in euthymic rats began with the onset of seroconversion. IgM antibodies against RV VP2 were detected by day 10, and IgG antibodies appeared by day 14. IgM titers were not detected by week 4, whereas IgG titers increased slowly (Table 6). The IgG response consisted primarily of IgG2a, the titers for which were consistently higher than for IgG1. IgG antibodies against RV NS proteins also were detected by 14 days and were present through week 8 (data not shown). Athymic rats developed weak anti-RV IgM responses beginning at day 14 but did not develop anti-RV IgG.

Perivascular accumulations of mononuclear cells appeared in tissues of euthymic rats by week 2, suggesting activation of cell-mediated immunity. They occurred in hepatic portal triads, adjacent to pulmonary vessels and mesenteric vessels, but were most conspicuous surrounding renal cortical vessels, where they continued to intensify through week 4 (Fig. 1C). Mononuclear cell infiltrates were not a feature of early infection in athymic rats, but mild perivasculitis developed among some renal and gonadal vessels and in a few hepatic portal triads by week 4. Several athymic rats also had developed mild bile duct hyperplasia.

DISCUSSION

Vasculotropism is an established feature of RV infection (5, 17, 20, 30, 38) and one that it shares with numerous other viruses (22). For most of these viruses endothelium is the prominent target. However, some vasculotropic viruses, such as encephalomyocarditis virus (12), human and murine herpesviruses (6, 42, 49, 56), and Seoul virus (R. O. Jacoby, S. R. Compton, F. X. Paturzo, and E. A. Johnson, unpublished data), have the capacity to infect SMC. A few early reports noted acute RV infection of SMC using routine histopathology (37, 38), and our prior ISH studies suggested that SMC support RV replication.
frequency of RV DNA-infected cells may reflect intracellular
nasia and tissue fixation. Further, the comparatively higher
levels of RV mRNA during the brief interval between eutha-
compared to that of DNA also may have reduced detectable
nucleus and easier to detect by ISH. The lability of mRNA
Additionally, one expects RV mRNA to be more dispersed
were below the level of ISH detection in some infected cells.
stage of virus replication (Ball-Goodrich et al., submitted)—
indicate that RV mRNA concentrations—which vary with the
needed to initiate parvoviral replication, this difference may
infection. Since comparatively small amounts of mRNA are
higher than for viral-mRNA-positive cells during all stages of
replication occurred during persistent infection, and explant
cultures confirmed the presence of infectious virus in persist-
tently infected kidney, lung, and spleen. Detection of virus by
ISH compared favorably with that by explant culture. Minor
discrepancies between the methods indicate, however, the
value of using complementary approaches to detect small
quantities of virus during persistent infection.
Virus-positive SMC were localized to subendothelial SMC
or dispersed throughout the muscle tunic. These patterns sug-
gest that SMC infection occurred by cell-to-cell extension from
overlying endothelium or through capillaries (vasa vasorum)
which supply blood to the muscle tunics. Although RV also
may have reached the SMC by penetrating between virus-
damaged endothelial cells, possibly adherent to red blood cells
(47), the absence of intravascular hemorrhage makes this path-
way less likely.

The results of PCNA staining support previous evidence that
SMC cycle in young adult rats (16). Thus, these cells meet an
important criterion for enabling RV replication. Furthermore,
vascular SMC in rats can proliferate in response to direct or
endothelial injury (14). Therefore, virus-induced endothelial
injury and subsequent infection of SMC may potentiate SMC
turnover and promote local RV infection. However, the pace of
cell-to-cell infection implicit to this possibility may be slow
enough to mask increased cell turnover in affected tissues.

The prevalence of viral-DNA-positive cells was consistently
higher than for viral-mRNA-positive cells during all stages of
infection. Since comparatively small amounts of mRNA are
needed to initiate parvoviral replication, this difference may
indicate that RV mRNA concentrations—which vary with the
stage of virus replication (Ball-Goodrich et al., submitted)—
were below the level of ISH detection in some infected cells.
Additionally, one expects RV mRNA to be more dispersed
intracellularly than RV DNA, which is concentrated in the
nucleus and easier to detect by ISH. The lability of mRNA
compared to that of DNA also may have reduced detectable
levels of RV mRNA during the brief interval between eutha-
nasias and tissue fixation. Further, the comparatively higher
frequency of RV DNA-infected cells may reflect intracellular
accumulation of nonreplicating virus (i.e., virus sequestration),
as has been hypothesized for persistent parvovirus infection of
mink. Mori and coworkers (44) found, in this regard, that viral
DNA in lymph nodes was prevalent among germinal center
cells resembling follicular dendritic cells or macrophages.
The presence of RV DNA, but not mRNA, in lymph node follicles
of persistently infected athymic rats resembles the results ob-
tained with mink. This distribution may represent sequestered
RV and/or concentration of scavenged RV DNA from persist-
ent infection in other tissues.

Viral mRNA was detected in PCNA-positive and PCNA-
negative SMC; suggesting that RV replication can proceed in
cells that are in the G0 or early G1 phase of the cell cycle. A
report by Lenghaus and coworkers offers some precedent for
this possibility (36). They demonstrated replication of feline
parvovirus in cultured cells in which DNA synthesis was
blocked by 6 mM thymidine. Although the mechanism was not
determined, they speculated that a cell function blocked by
thymidine may have been assumed by a viral protein or that
part of an infected cell’s DNA-replicating machinery was suf-
ficient and available to support parvoviral replication. Never-
theless, other explanations must be explored before concluding
that active RV replication occurs in cells that are not in S
phase. For example, cell death may not be the sole outcome of
RV replication during a single pass of infected SMC through
the cell cycle. If viral transcription or replication does not
attain peak levels in SMC prior to the end of S phase, it may
be present when the cell returns to G0, an interval when PCNA
is not expressed.

Technical deficiencies also must be considered to explain the
variable presence of PCNA in SMC containing RV mRNA.
Inadequate sensitivity of the immunostaining method for
PCNA is an unlikely factor, since the results were at least as
sensitive as those reported previously for rat tissues (19, 21).
However, the intensity of PCNA staining varies during the cell
cycle (21), with weaker reactions expected during G1 or early S
phase. Therefore, small amounts of PCNA may not have been
detected by standard light microscopy. In this context, hybrid-
ization with the radiolabeled riboprobe may have quenched
detection of PCNA, producing false-negative results. However,
the fraction of PCNA-positive cells was approximately the
same in tissues that were labeled for PCNA and mRNA as in
those stained only for PCNA. Furthermore, the ratios of cells
which stained strongly versus weakly for PCNA in the two
groups of tissues were similar. The lack of a definitive expla-
nation for RV mRNA signal in PCNA-negative SMC justifies
closer examination of RV replication in such cells. Infection of
synchronized SMC in vitro, including staining for cell cycle

<table>
<thead>
<tr>
<th>Week (no. of rats)</th>
<th>Antibody</th>
<th>Titer in an individual rat (10^6)</th>
<th>Mean titer ± SD</th>
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<td>7.81 ± 3.13</td>
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<td>3.5 ± 3.2</td>
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<tr>
<td></td>
<td>IgG2a</td>
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<td>Ig</td>
<td>6.25 6.25 12.5 6.25 6.25 6.25</td>
<td>7.29 ± 2.56</td>
</tr>
<tr>
<td></td>
<td>IgG1</td>
<td>0.25 0.25 0.25 1.25 0.25 0.25</td>
<td>1.42 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>IgG2a</td>
<td>6.25 1.25 6.25 6.25 6.25 6.25</td>
<td>5.42 ± 2.04</td>
</tr>
<tr>
<td>8 (6)</td>
<td>Ig</td>
<td>25.0 12.25 25.0 25.0 25.0 50.0</td>
<td>27.08 ± 12.29</td>
</tr>
<tr>
<td></td>
<td>IgG1</td>
<td>1.25 1.25 1.25 1.25 1.25 12.5</td>
<td>2.96 ± 4.69</td>
</tr>
<tr>
<td></td>
<td>IgG2a</td>
<td>25.0 12.5 25.0 25.0 12.5 50.0</td>
<td>25.0 ± 13.69</td>
</tr>
</tbody>
</table>

* Each column indicates titers for an individual rat.
proteins less generic than PCNA, may clarify whether RV replication can progress in noncyling cells.

The comparison between euthymic and athymic rats confirmed that the intensity of persistent infection is strongly influenced by host immunity. The reduction in the number of virus-infected cells after seroconversion in euthymic rats is consistent with a role for humoral immunity. However, the development of mononuclear cell infiltrates in infected tissues in our study and in prior investigations (24, 30) and the prominence in this study of IgG2a responses, consistent with a Th1 response in the rat (33), justify exploration for cell-mediated responses. Weak IgM and mononuclear responses occurred in the athymic rats. This result was not surprising given the proclivity of athymic rats to develop some T cells as they age (57). Additionally, athymic rats are capable of producing IgM responses to thymus-independent antigens (60), which may mean that RV has at least one epitope of this type.

The onset of immunity did not eliminate infection in euthymic rats but may promote the sequestration of RV and/or reduce virus replication. These possibilities are consistent with the results of passive immunization, wherein RV immune serum, administered to infected juvenile athymic rats, transiently suppressed detection of infectious virus (23). Antiviral immunity also may reduce cell-associated expression of viral proteins, impeding effective immune recognition of infected cells (1, 46). Alternatively, initial exposure to RV prior to immunologic maturity may result in delayed elimination of virus due to suboptimal immune responses. Immunologic maturation in rats is not complete until at least 1 month after birth (3, 43, 59, 61). Therefore, the 6-day-old rats used in this study were presumably immuno- suppressed when they were inoculated. Additionally, RV may retard anti-RV immunity because it is at least transiently immunosuppressive (40). We are currently pursuing the influence of anti-RV immunity further by determining responses to virus and viral proteins in adult rats.

Viral persistence is a feature of infection caused by other autonomous parvoviruses (15, 48, 55, 58). Among these, the most detailed pathogenesis studies have been performed with Aleutian disease virus (ADV) (2, 9, 10, 39). Persistent RV and ADV infection share the properties of low-level infection after infection of the mouse aorta: an ultrastructural study. Am. Heart J. 86:669–705.


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