

Infection of Peripancreatic Lymph Nodes but Not Islets Precedes Kilham Rat Virus-Induced Diabetes in BB/Wor Rats

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A parvovirus serologically identified as Kilham rat virus (KRV) reproducibly induces acute type I diabetes in diabetes-resistant BB/Wor rats. The tissue tropism of KRV was investigated by in situ hybridization with a digoxigenin-labelled plasmid DNA probe containing approximately 1.6 kb of the genome of the UMass isolate of KRV. Partial sequencing of the KRV probe revealed high levels of homology to the sequence of minute virus of mice (89%) and to the sequence of H1 (99%), a parvovirus capable of infecting rats and humans. Of the 444 bases sequenced, 440 were shared by H1. KRV mRNA and DNA were readily detected in lymphoid tissues 5 days postinfection but were seldom seen in the pancreas. High levels of viral nucleic acids were observed in the thymus, spleen, and peripancreatic and cervical lymph nodes. The low levels of infection observed in the pancreas involved essentially only endothelial and interstitial cells. Beta cells of the pancreas were not infected with KRV. These findings suggest that widespread infection of peripancreatic and other lymphoid tissues but not pancreatic beta cells by KRV triggers autoimmune diabetes by perturbing the immune system of genetically predisposed BB/Wor rats.

The Biobreeding/Worcester (BB/Wor) rat provides valuable models for both spontaneous and inducible insulin-dependent (type I) diabetes mellitus. Spontaneous type I diabetes mellitus occurs in >80% of virus antibody-free rats of the lymphopenic diabetes-prone strains, usually between 50 and 120 days of age (24, 31). Susceptibility has been associated with environmental (20) as well as major histocompatibility complex and non-major histocompatibility complex genetic factors (7, 15). The closely related BBDR/Wor diabetes-resistant (DR) strain has a normal complement of lymphoid subsets and is resistant to the development of spontaneous diabetes (20). However, diabetes can be induced in DR rats under certain experimental and environmental conditions (13, 34, 38).

A viral model for acute autoimmune diabetes in the DR rat was recently developed (14). Discovery of this model followed two natural epidemics of diabetes among two colonies of DR rats (19, 38). A virus tentatively identified by serology as Kilham rat virus (KRV) was cultured from the pancreas, plaque purified, and subsequently shown to induce acute insulinitis and diabetes 2 to 4 weeks after inoculation of 21- to 25-day-old DR rats. This viral isolate has been named KRV-UMass. KRV titers in tissues and blood peaked at 3 to 5 days postinoculation, with titers in the pancreas decreasing 2 to 3 logs from day 5 to day 10 postinoculation (14). Distinct from other models of virus-induced diabetes (26, 41), evidence of KRV infection of the pancreatic beta cells was not found by immunostaining either at the peak of infection or at the time of insulinitis (14), and by the time of onset of diabetes, KRV antigens could no longer be detected in endothelial or interstitial cells of the pancreas (unpublished observations).

KRV is a parvovirus, and parvoviruses require replicating cells going through the S phase of the cell cycle to complete their infectious cycle (37). Tropism plays a key role in

determining the outcome of infection of the host (35), as exemplified by the distinct tropisms and pathologies associated with neonate versus weanling kittens and puppies infected with feline parvovirus (5, 17) and canine parvovirus (22, 30), respectively. In view of the fact that all previously reported animal models of virus-induced diabetes involved direct viral infection of beta cells (26, 41), it was critical that a highly sensitive assay be used to screen pancreatic islets for KRV gene products. In the present study, we present a preliminary molecular analysis that demonstrates that the UMass isolate is indeed a parvovirus, and we evaluate its tissue tropism by in situ hybridization with a double-stranded DNA probe capable of detecting both positive- and negative-sense viral DNA as well as mRNA.

MATERIALS AND METHODS

Cloning of a partial genomic clone of KRV-UMass. The double-stranded replicative form of KRV DNA was isolated from KRV-UMass-infected normal rat kidney (NRK) cells by a modified Hirt procedure as described by Molitor et al. (23). The DNA was isolated in a 1% agarose gel in TAE buffer (40 mM Tris-acetate [pH 8.3], 20 mM Na-acetate, 2 mM EDTA), and the 5-kb band was extracted by binding to a silica matrix (Geneclean, Bio 101, Inc., La Jolla, Calif.). Conserved restriction endonucleases were selected on the basis of known sequence information from two other parvoviruses (minute virus of mice [MVM] and H1). An *Eco*RI and *Hind*III double digest was used to obtain an approximately 1.6-kb internal fragment corresponding to nucleotides 1087 to 2653 and 1085 to 2651 of the genomes of H1 (29) and MVM (2), respectively. This fragment was then inserted into the Bluescript KS⁻ vector (Stratagene, La Jolla, Calif.) and selected on the basis of blue-white color selection on X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside)-containing L broth agar plates.

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Sequence analysis of the KRV probe. In order to confirm the identity of the region of the genome cloned, 444 bases at the 5' end of the plus strand were sequenced with a modification of the Sanger chain-termination sequence reaction (39). The sequence was then compared with that of other parvoviruses by the align program of DNASTAR (DNA STAR, Inc., Madison, Wis.).

Labelling of the probe. The KRV probe was labelled in a reaction mix containing 1× nick translation buffer and enzyme mix (GIBCO-BRL, Gaithersburg, Md.), 1 µg of silica matrix-purified probe DNA, and 60 µM (each) of dATP, dCTP, dGTP, and dig-11-dUTP (Boehringer Mannheim, Indianapolis, Ind.). After 3 h at 15°C, the labelled DNA was ethanol precipitated in the presence of salmon sperm DNA and *Escherichia coli* tRNA and resuspended in distilled H₂O at 10 ng of probe DNA per µl.

Infection of rats and NRK cells. Virus antibody-free BBDR/Wor rats were obtained from the research- and National Institutes of Health contract-supported colonies located at the University of Massachusetts Medical Center. DR rats of both sexes (21 to 25 days of age) were inoculated intraperitoneally with 0.1 ml of a 1:6 dilution (approximately 5×10^7 PFU) of a defined KRV stock (National Cancer Institute [NCI] T953000) (NCI KRV) provided by W. Shek (Charles River Laboratory, Wilmington, Mass.).

NRK cells were grown to subconfluency on microscope slides, infected with the UMass isolate of KRV, and fixed the following day as described above. Both the UMass and NCI isolates of KRV induce diabetes in young virus antibody-free BBDR/Wor rats (14).

Fixation of tissues. Rats were killed 5 days postinoculation, and tissues were snap frozen in OCT (Miles Inc., Elkhart, Ind.) by immersion in liquid nitrogen-cooled isopentane and stored at -20°C. Four-micrometer-thick sections of tissues were prepared with a cryostat kept at -29°C. Cryostat sections and NRK cells grown on slides were immersed in fresh 4% paraformaldehyde in phosphate-buffered saline (PBS) (pH 7.5) for 5 min at room temperature. The fixed slides were then transferred to ice-cooled 70% ethanol and maintained at -20°C until used in hybridization studies.

In situ hybridization. Lyophilized probe was resuspended in 100% formamide (10 ng of probe DNA per µl of formamide) and denatured by heating at 75°C for 5 min. An equal volume of hybridization buffer containing 2 parts dextran sulfate, 1 part 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1 part bovine serum albumin (BSA) (20 mg/ml), and 1 part vanadyl ribonucleoside complex (GIBCO-BRL) was added to the probe essentially as described by McNeil et al. (21). The probe solution was applied to the cells or section being evaluated and then covered with parafilm and incubated overnight at 37°C in a humidity chamber. Following successive washes in SSC-containing solutions (21), the cells or sections were covered with a 1:20 dilution of peroxidase-labelled antidigoxigenin antibody (Boehringer Mannheim) in 1% BSA in PBS, incubated for 1 h at 37°C, and rinsed several times in 4× SSC (with and without Triton X-100) (21). Bound peroxidase-labelled antibody was detected by staining in 0.04% diaminobenzidine-0.012% H₂O₂. Hematoxylin was used as a counterstain.

Fixation and staining protocols for immunohistological identification of KRV antigen were performed as previously described (14).

Nucleotide sequence accession number. The 444-bp partial nucleotide sequence of KRV-UMass has been deposited with GenBank under the accession number L20503.

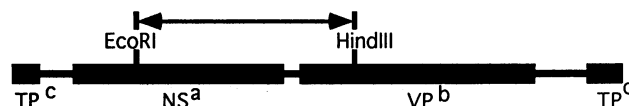


FIG. 1. Region of KRV-UMass cloned into Bluescript KS⁻. The genome of closely related H1 is shown above, indicating open reading frames encoding the nonstructural proteins NS1 and NS2 and virion proteins VP1 and VP2. The *Eco*RI-to-*Hind*III region of KRV double-stranded replicative form DNA corresponds to nucleotides 1087 to 2653 of H1. a, open reading frame encoding NS1 and NS2, nonstructural proteins required for transcription and DNA replication; b, open reading frame encoding the viral coat proteins; c, terminal palindromes.

RESULTS

Confirmation of probe specificity. Although KRV is the first parvovirus to have been isolated, only a small portion of the virus has been cloned and sequenced (1, 32). We therefore cloned part of the UMass KRV isolate. (This region does not overlap with that previously cloned and sequenced.) Figure 1 is a diagram of the region of KRV-UMass cloned into the Bluescript KS⁻ vector. Assuming a genome similar to that of the H1 and MVM parvoviruses, the insert consists primarily of the region encoding the nonstructural proteins NS1 and NS2. This region of the genome is more highly conserved among parvoviruses than that which encodes the viral capsid proteins. KRV-UMass sequence information is provided in Fig. 2. Bases which differ from the H1 or the MVM sequence are indicated. Sequence analysis of the KRV probe revealed a very high degree of homology (99%) with the H1 genome. A total of 440 of 444 bases were identical (Table 1). High homology (89%) was also observed between KRV and MVM. Other parvoviruses had considerably lower levels of homology to KRV in this region. A second subgenomic clone of KRV was produced by inserting a *Dra*I internal fragment of NCI KRV double-stranded replicative form DNA into Bluescript KS⁻. Within the 444 bases illustrated in Fig. 2, 200 bases of the NCI KRV isolate were sequenced and found to be 100% homologous to KRV-UMass (data not shown). These results support the serological identification of the UMass isolate as KRV.

Figure 3A illustrates in situ results with the digoxigenin-labelled KRV-UMass subgenomic probe to detect KRV-UMass-infected NRK cells 23 h after infection. Infected and uninfected cells of the infected NRK culture are easily

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GAATTCAAACtAgAAAgGAGGTcTCgATTAAACcACACTcAAAGAGtGGTGcATAA
AAGAGTAACCTCACCAGaGACTGGATGATGATGCAGCCAGACAGTtACATTGAAA
TGATGGCTCAACCAGGTGGAGAAAACtGCTtAAAATACaCTAGAGATcTGTACACT
gACTCTAGCaAGAACCAAAACAGCcTTTGACTTgATTcTgGAAAAAGCTGAAACCAGC
AAACTAgCCAACtTTTCaTGgCTAgCACcAGAACCTGtAGAAATcTTTGCTgagCATGGCT
GGAACTATaTTAAAGTcTGCCATGCcATcTGtGTGTgTgAAIAGACAAGGAGGCAAA
AGGAACACTGTgTcTtTTTCaCGGACCAGCCAGCACAGGCAAACTATcATTGCACAAG
CCATAGCACAAGgAGTTGGtAATGTTGGTTGtATcA
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FIG. 2. Partial sequence of KRV-UMass probe. The sequence is for the positive strand of the double-stranded replicative form of KRV extending 3' from the internal *Eco*RI site. Four nucleotides that differ from the H1 sequence (29) are highlighted with enlarged boldface lettering. Fifty-one nucleotides differing from the MVM sequence (2) are indicated by lowercase lettering. GenBank accession number, L20503.

TABLE 1. Comparison of KRV with other parvoviruses for sequence homology in a 444-base region extending from the *EcoRI* site of the KRV probe^a

Viruses being compared	Similarity index (%)	No. of matched bases	No. of gaps
KRV vs H1 ^b	99	440	0
KRV vs MVM	89	393	0
KRV vs CPV ^c	77	340	0
KRV vs FPV ^d	76	339	0
KRV vs MEV ^e	76	338	0
KRV vs PPV ^f	73	326	3
KRV vs ADV ^g	59	248	22
KRV vs B19 ^h	54	224	24
KRV vs BPV ⁱ	51	204	19

^a Sequence information for the selection of parvoviruses compared with KRV was obtained from GenBank. Comparisons were made with the align program of DNASTAR (DNASTAR, Inc.) with a K-tuple size of 3, a range of 20, and a gap penalty of 3. For viruses with lower homology to KRV (Aleutian disease virus of mink, B19, and bovine parvovirus), comparisons were restricted to the NS-encoding region of the genome.

^b A rodent parvovirus capable of infecting humans (29).

^c Canine parvovirus (28).

^d Feline parvovirus (27).

^e Mink enteritis virus (16).

^f Porcine parvovirus (40).

^g Aleutian disease virus of mink (3).

^h An autonomous human parvovirus (33).

ⁱ Bovine parvovirus (6).

distinguished. Although the in situ staining is primarily nuclear, consistent with the nuclear replication of parvoviruses, cytoplasmic staining is also evident. Since the positive- and negative-sense strands of the probe were labelled, both genomic DNA and mRNA of the virus should be detected.

Localization of virus in the host. Figures 3B to F illustrate representative cryostat sections of tissues from KRV-infected rats 5 days after inoculation. As in Fig. 3A, the sections had been hybridized to a digoxigenin-labelled KRV probe. KRV's preference for lymphoid tissue is evident in Fig. 3B, C, and D, which illustrate sections of KRV-infected spleen, thymus, and cervical lymph node, respectively. Megakaryocytes are one of the major cells infected in the spleen, an observation made earlier with immunohistochemistry (14). The staining pattern observed in the thymus (Fig. 3C) is variable concerning the number of positive cells in the medulla and cortex. This may reflect the time course of viral spread. The medullary localization of virus-positive cells might represent an early stage of thymus infection, since more heavily infected thymi are characterized by a high frequency of virus-positive cells in the cortex. Numerous cells in the cervical lymph node (Fig. 3D) stain positively for KRV nucleic acids, whereas the adjacent salivary gland appears free of infection. In the cryostat section of the pancreas and peripancreatic lymph node illustrated in Fig. 3E, a moderately heavy reaction product is seen in the peripancreatic lymph node, whereas staining of the pancreas is very limited. Of 20 pancreas sections evaluated (from 20 animals, approximately nine islets per section), only one islet revealed minimal evidence of infection (a single positive cell of uncertain phenotype). The few infected cells observed in the exocrine pancreas were primarily endothelial and interstitial cells (not shown). Representative of a typical islet, the pancreatic islet shown in Fig. 3F is free of infection. An immunoperoxidase preparation of small intestinal mucosa (not shown) revealed a greater concentration of virus (antigen) in an adjacent lymphoid nodule. Intraepithelial

lymphocytes and intestinal epithelial cells were also sites of viral replication.

DISCUSSION

Partial sequence analysis of the region encoding the non-structural proteins revealed a high level of homology between the UMass isolate of KRV and H1 and a high but decreased degree of homology between the UMass isolate of KRV and MVM. This sequence information proves decisively that the diabetes-inducing virus is a parvovirus and not another virus type serologically cross-reactive with KRV. Although further sequence analysis to determine whether H1 and KRV retain high homology throughout the genome is in progress, it is clear that their capsid proteins are serologically distinct (8).

Previous results (14) and the information presented in this communication support the hypothesis that KRV induces diabetes in BBDR/Wor rats by triggering a genetically programmed anti-beta cell immune response, rather than by directly infecting and destroying pancreatic beta cells. How might the virus trigger the pathologic immune response? One possible mechanism could involve molecular mimicry, as has been previously described for other viral systems (11, 25, 36). If such a mechanism were operative, it would require the presence of a cross-reactive epitope shared by KRV and the beta cells of DR rats. This epitope, however, would be unable to elicit an anti-beta cell response in rats other than BBDR/Wor, since KRV does not induce diabetes in diabetes-resistant strains such as Wistar Furth, PVG, and Long Evans (14). A low level of cross-reactivity that would normally be of little consequence might be sufficient to trigger an anti-islet response in individuals with the appropriate susceptibility to autoimmune diabetes.

A second mechanism would be an alteration of subsets of lymphoid cells and the subsequent compromise of immunoregulation. The efficient replication of virus in lymphatic tissues would support such a mechanism. A low frequency of KRV-induced lymphocytic thyroiditis (unpublished observations) also suggests a generalized defect in immunoregulation. It has recently been reported that an RT6⁺ CD4⁺ T-cell subset inhibits autoimmune diabetes induced in normal rats following adult thymectomy and sublethal gamma irradiation (10). However, analysis of peripheral blood T-cell subsets (including lymph node RT6⁺ T cells) after infection of BBDR/Wor rats with KRV failed to reveal significant changes in the percentages of peripheral blood T-cell subsets (14).

A third mechanism could involve a viral or cytokine influence on the vascular permeability of the pancreas. Both DR and diabetes-prone BB/Wor rats are characterized by increased pancreatic vascular permeability to the colloidal pigment Monastral blue B (9). Enhanced vascular permeability might lead to increased exposure of beta cell antigens to the lymphoid system and increased exposure of islet cells to immune attack. KRV-infected endothelial cells were observed. Although these cells were infrequently observed in the pancreas, it is not known what magnitude of endothelial infection significantly alters vascular permeability or whether cytokines induced by viral infection would contribute to the effects on the vasculature. Viral infection of the pancreas, however, is not sufficient for insulinitis induction, since vaccinia virus efficiently infects the exocrine portion of the BBDR/Wor pancreas yet fails to induce either diabetes or insulinitis (unpublished observations). Also, KRV does not induce diabetes or insulinitis in diabetes-prone rats within 4

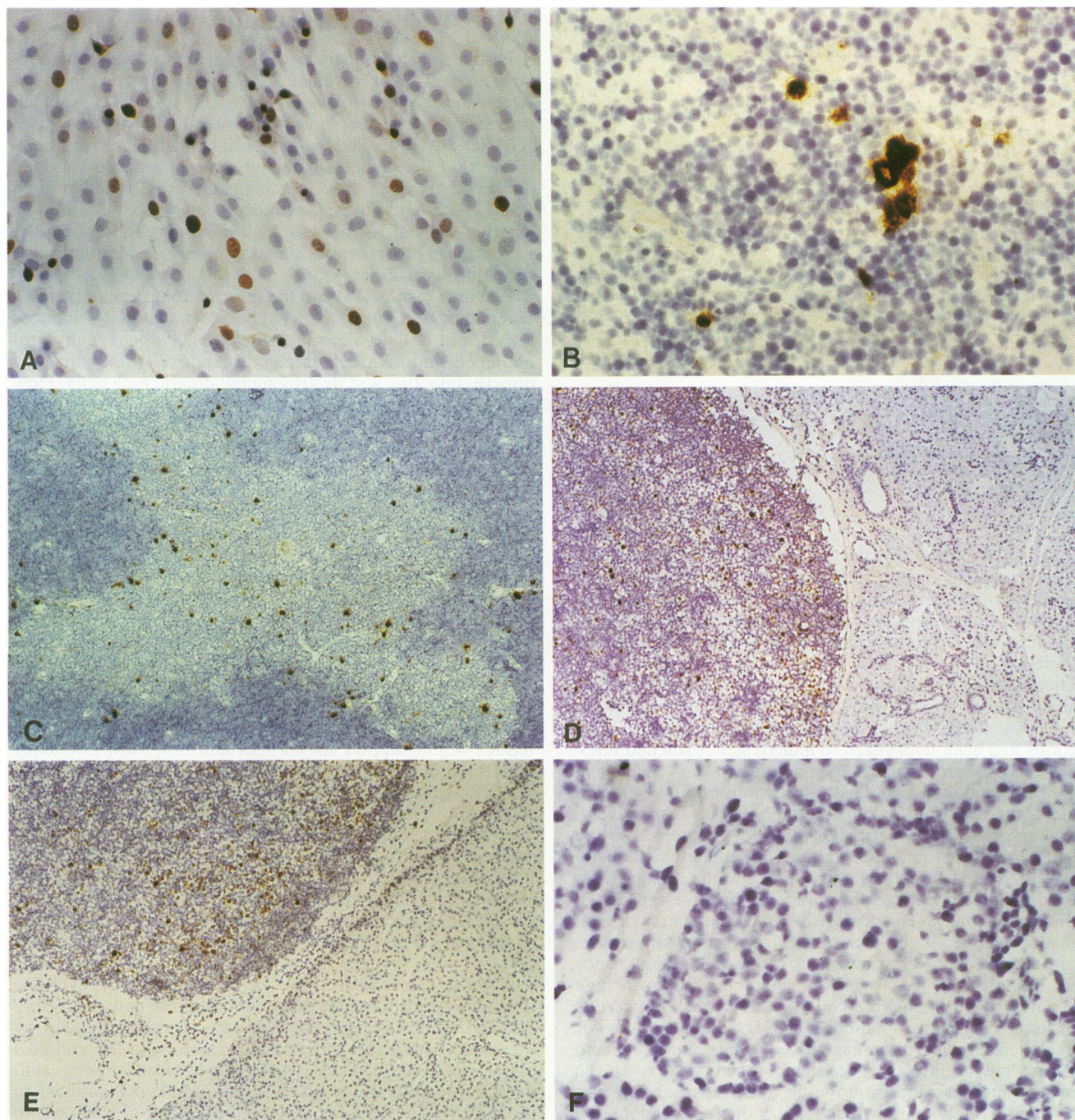


FIG. 3. Photomicrographs of in situ hybridization for the presence of KRV mRNA and genomic DNA. The presence of viral nucleic acids is indicated by a dark brown reaction product and is consistent with the nuclear replication of KRV. Host tissues (B to F) were collected 5 days postinoculation. (A) NRK cells 23 h after infection with KRV. Magnification, $\times 120$. (B) Infected megakaryocytes and mononuclear cells in the spleen of a KRV-infected BBDR/Wor rat. Magnification, $\times 250$. (C) Thymus of a KRV-infected DR rat illustrating a predominantly medullary distribution of virus-positive cells. Magnification, $\times 62$. (D) Cervical lymph node and salivary gland of KRV-infected DR rat. In spite of heavy infection of the adjacent lymph node (left side of micrograph), the salivary gland appears free of infection. Magnification, $\times 62$. (E) Peripancreatic lymph node (upper left) and pancreas of KRV-infected DR rat. Magnification, $\times 62$. The lymph node reveals significant infection. At this magnification, the pancreas appears free of reaction product. (F) Typical pancreatic islet from KRV-infected DR rat showing no evidence of infection. Magnification, $\times 250$.

weeks of infection, unless the animals have been initially reconstituted with DR spleen cells (14). Cytokines might directly affect pancreatic beta cells by upregulating surface major histocompatibility complex antigen expression (4) or by the production of potentially destructive levels of nitric oxide by nonendocrine islet cells (18).

A fourth possibility, compatible with the results of the present study, is that viral replication in the draining lymph nodes of the BB/Wor pancreas might trigger genetically autoreactive cells residing therein. The large number of virus-positive cells observed in the peripancreatic lymph nodes and other lymphoid tissues are likely to trigger a vigorous antiviral response, and lymphokines released could potentially activate T cells specific for nonviral antigens. Lymph nodes of genetically susceptible animals might have an abundance of autoreactive cells capable of responding to immunological signals provided by the antiviral response.

Analyses of pancreatic lysates for titers of KRV had previously suggested a significant infection of the pancreas (14). Peripancreatic lymph nodes had not been removed from the titrated pancreatic tissue samples, and the present results suggest that lymph nodes were the major source of virus detected in the pancreas. Gaertner et al. (12) recently characterized the distribution of rat parvovirus in Sprague-Dawley rats by *in situ* hybridization and found no evidence of infection of islet cells, although they did observe infection of pancreatic interstitial cells and occasional acinar cells. The tissue and serum titers of virus observed were greater in both magnitude and duration than we reported with BBDR/Wor rats and likely reflect the young age of the rats at the time of Gaertner's inoculations (2 days of age).

KRV-induced diabetes in the BB/Wor rat thus provides a valuable model for virus-induced autoimmunity in a genetically susceptible host. KRV's ability to trigger an anti-islet cell response in these rats could potentially mirror a similar etiology for type I diabetes in humans.

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