tris-HCl, pH 7.5, 20 mM NaCl, and 1 mM EDTA. Precipitated lipids were removed by centrifugation, and the clarified solution was passed over a Sepharose FAST Q column. The nonretained fractions were pooled and directly applied to a Sepharose FAST S column. Pure enzyme (>98%) lacking lipid contaminants was eluted from this column with 1 M NaCl and 50 mM Hepes, pH = 7.5, and dialyzed into the crystallization buffer. The crystals of the uninhibited form of hnps-PLA_2 (0.8 mm by 0.4 mm; $P6_122$; $a = b = 76.3$ Å, and $c = 90.6$ Å; one molecule in the asymmetric unit) grew in 5 days at 4°C from 10-μl droplets containing 10 mg/ml protein, 5 mM $CaCl_2$, 0.1 M tris, 2.5 M NaCl, 0.5 mM β-octyl-glucopyranoside, pH = 7.4, that were plated onto plastic cover slips and sealed over reservoirs containing 1 ml of 4.9 M NaCl, 0.1 M tris, pH = 7.4. Two different crystal forms of hnps-PLA_2–TSA were characterized. Crystals of type 1 (0.4 mm by 0.2 mm by 0.2 mm; $P6_322$; $a = b = 64.9$ Å, $c = 113.8$ Å; one molecule in the asymmetric unit) were grown in 2 weeks at room temperature from 20-μl droplets containing 10 mg/ml protein, 10 mM $CaCl_2$, 0.1 M tris, 2.0 M NaCl, pH 7.4, that were plated onto glass depression slides and sealed in boxes containing 15 ml of 4.0 M NaCl, 0.1 M tris, pH = 7.4. The crystallization of Type 2 crystals (0.5 mm by 0.5 mm by 0.4 mm; $P4_32_12$; $a = b = 76.3$ Å, $c = 115.3$ Å; two molecules in the asymmetric unit) differed from the preceeding only in that a higher NaCl concentration was used (2.25 and 4.5 M in the droplet and reservoir, respectively). Crystals of the uninhibited enzyme yielded a diffraction data set that extended to 2.2 Å resolution (R_{sym} = 0.085), Type I crystals provided data to 2.8 Å resolution (R_{sym} = 0.142), and Type II crystals diffracted to 2.1 Å resolution (R_{sym} = 0.080). All data were collected from single crystals with a dual panel San Diego Multiwire System detector; source radiation was graphite-monochromated CuKα emission from an RU-300 x-ray generator.

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39. The coordinates will be deposited in the Brookhaven Protein Data Bank. The research at Yale was supported by NIH grant no. GM22324 and by the Howard Hughes Medical Institute; the research at the University of Washington was supported by NIH grant no. HL36235. S.P.W. was a fellow of the Arthritis Foundation, and D.L.S. was a graduate student at the University of Chicago and a postgraduate fellow at Yale. We thank Z. Otwinowski for assistance in data collection, and W. Meier and K. Miatkowski for their help with large-scale culturing of the CHO line.

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Induction of Type I Diabetes by Kilham's Rat Virus in Diabetes-Resistant BB/Wor Rats

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Type I diabetes mellitus is an autoimmune disease resulting from the interaction of genetic and environmental factors. A virus that was identified serologically as Kilham's rat virus (KRV) was isolated from a spontaneously diabetic rat and reproducibly induced diabetes in naive diabetes-resistant (DR) BB/Wor rats. Viral antigen was not identified in pancreatic islet cells, and β cell cytolysis was not observed until after the appearance of lymphocytic insulitis. KRV did not induce diabetes in major histocompatibility complex–concordant and discordant non-BB rats and did not accelerate diabetes in diabetes-prone BB/Wor rats unless the rats had been reconstituted with DR spleen cells. This model of diabetes may provide insight regarding the interagation of viruses and autoimmune disease.

VIRAL INFECTIONS HAVE BEEN considered possible etiologic agents responsible for human type I diabetes with the coxsackie (*1, 2*) and rubella (*3*) viruses as the most likely candidates. Proposed mechanisms of action include virus-induced modification of β cell antigens, molecular mimicry, direct lysis of β cells, and virus-induced functional changes in effector or regulatory lymphocytes (*4, 5*). All experimental models of virus-induced diabetes so far involve viral infection or transfection of β cells, and there has been no direct demonstration that viruses induce diabetes in experimental animals without extensive β cell infection or lysis (*4–6*). We report here a model for the study of virus-induced diabetes in rats with a discrete genetic background.

The BB/Wor rat develops spontaneous, autoimmune diabetes mellitus and is considered an animal model of human type I insulin-dependent diabetes mellitus. Diabetes-prone (DP) rats are lymphopenic and >80% develop diabetes before 120 days of age (*7, 8*). DR rats were derived from DP progenitors (*9*), have normal lymphocyte numbers and phenotypes, and do not become diabetic spontaneously if maintained in a viral antibody–free (VAF) environment (*10*).

An unexplained outbreak of lymphocytic insulitis (pancreatic islets surrounded and

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permeated by lymphocytes) and hyperglycemia in the University of Massachusetts (UMass) Medical Center colony of DR rats occurred in 1984 to 1986 (*11*). Although diabetes frequently occurred in clusters, we assumed a genetic explanation and attempted to produce family lines of diabetic DR rats. We produced >500 progeny from a variety of mating schemes, including animals produced by crossing parents that were both diabetic and nonlymphopenic. Less than 1% of these animals became diabetic, and the crosses provided no clear genetic mechanism for diabetes in DR rats. Serologic data (*12*) collected from 1984 to 1988 suggested an association between the presence of antibodies to the common rat parvovirus Kilham's rat virus (KRV) and these diabetic rats (Fig. 1). In 1989 to 1990, another outbreak of diabetes occurred in a satellite colony of BB/Wor DR rats housed in a conventional (non-VAF) animal room. Twenty-nine diabetics were detected during a 60-day period in a room housing approximately 350 DR rats, and serologic profiles indicated the presence of antibodies to KRV, as well as Toolan's H-1, sialodacryoadenitis (SDAV), and Sendai viruses (*13*). During the 1989 outbreak, we isolated viruses from the spleens, lymph nodes, bone marrows, and pancreases of several diabetic and nondiabetic DR rats (*14*). We plaque-purified a pancreas isolate, serologically identified it as KRV (*15*), and used it to test the hypothesis that KRV infections induce lymphocytic insulitis and diabetes mellitus in DR BB/Wor rats.

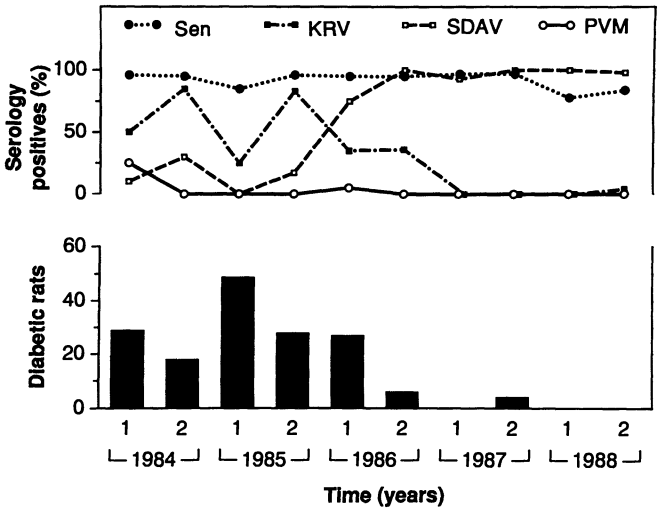

Fig. 1. Serology and spontaneous diabetes in BB/Wor DR rats from 1984 to 1988. (**Top**) Serum samples from 10 to 20 rats were tested for viral antibodies (*12*), and the results were graphed as the percent of positive tests for 6-month intervals by year. (**Bottom**) The number of spontaneously diabetic DR rats detected in the colony during the same 6-month periods. Total number of DR rats in the colony varied from 750 to 1000. Sen, Sendai virus.

Table 1. Frequency of diabetes or insulitis in rats injected with KRV or SDAV. Intact litters of 21- to 25-day-old male and female rats were inoculated intravenously or intraperitoneally with 0.2 ml of KRV-UMass containing 1.4×10^5 pfu or intranasally or intraperitoneally with 0.1 ml of SDAV (CRL-V22) containing 100 median tissue culture infectious doses. Vehicle inocula consisted of similarly diluted tissue culture fluid from uninfected NRK cells. Animals were tested for diabetes until 50 days of age. Diabetic animals (*16*) were killed at detection, and nondiabetics were killed at 50 days of age for morphologic examination of pancreatic tissues and collection of sera for viral serology. The presence of lymphocytic insulitis was evaluated by an observer without knowledge of the identity or status of the experimental animals. The results represent pooled data from 15 separate experiments. WF, Wistar Furth rats; DR spleen; spleen cells from DR rats.

Rat	Treatment	Diabetes	Insulitis*
BB/Wor DR ($RT1^{u/u}$)	KRV-UMass	40/129	39/82
	NCI KRV	10/30	9/20
	SDAV	0/37	0/37
	Vehicle	0/63	3/57†
	No injection	0/59	3/56†
BB/Wor DP ($RT1^{u/u}$)	KRV-UMass	0/20	3/20
	DR spleen cells	0/10	1/9†
	DR spleen + KRV	12/20	5/8
	Vehicle	0/20	0/20
	DR spleen + vehicle	0/10	4/10†
WF ($RT1^{u/u}$)	KRV-UMass	0/20	0/20
Long Evans ($RT1^{u/u}$)	KRV-UMass	0/15	0/15
PVG-R^8 ($RT1^{a/u}$)	KRV-UMass	0/40	0/40
PVG-R^{23} ($RT1^{u/a}$)	KRV-UMass	0/10	0/10
PVG ($RT1^{c/c}$)	KRV-UMass	0/19	0/19

*Lymphocytic insulitis without diabetes. †The rats with insulitis were KRV seropositive.

Table 2. KRV titers in tissues and blood. Blood and organ suspensions were titrated by plaque assay of NRK cells. We incubated plaque-assay plates for 5 days before staining them with crystal violet and counting the plaques. Values are geometric mean titers ± SE of the logarithm of the pfu of a liver lobe, pancreas, or spleen or the logarithm of the pfu per milliliter of blood. A less than sign (<) indicates that at least one sample had titers below the limit of the assay and that this low-limit value was incorporated into the calculation of the mean.

Tissues	Days after infection			
	3	5	7	10
Pancreas (n = 5)	6.8 ± 0.2	6.5 ± 0.2	5.6 ± 0.3	4.0 ± 0.5
Liver lobe (n = 5)	7.0 ± 0.1	7.4 ± 0.2	7.3 ± 0.2	6.2 ± 0.2
Spleen (n = 5)	8.1 ± 0.1	7.5 ± 0.1	6.0 ± 0.1	5.1 ± 0.3
Blood (n = 4)	3.0 ± 0.1	3.0 ± 0.2	<2.1 ± 0.1	<2.0 ± 0

We inoculated 21- to 25-day-old VAF DR and DP rats intravenously or intraperitoneally with the KRV-UMass isolate. Thirty-one percent of the DR rats developed acute, ketosis-prone diabetes within 2 to 4 weeks after KRV injection (*16*). An additional 30% demonstrated lymphocytic insulitis without diabetes. Although KRV induced diabetes or insulitis in 61% of the DR rats, it did not induce either condition in young DP rats within 4 weeks of infection unless the animals had been initially reconstituted with DR spleen cells (*17*). DR rats inoculated with a defined KRV seed stock [National Cancer Institute (NCI) T953000] (NCI KRV) obtained from the Charles River Laboratory (CRL) showed similar frequencies of diabetes and insulitis (Table 1).

Diabetes susceptibility in BB rats is associated with genes that encode the $RT1^u$ major histocompatibility complex (MHC) haplotype (*18*). DP and DR BB/Wor rats are both $RT1^{u/u}$. To determine whether the u haplotype is sufficient for KRV-induced diabetes, we injected Wistar Furth ($RT1^{u/u}$), Long Evans ($RT1^{u/u}$), PVG ($RT1^{c/c}$), and congenic PVG-R^8 ($RT1^{a/u}$) and PVG-R^{23} ($RT1^{u/a}$) rats (*16*) with the same quantity of KRV-UMass used to induce diabetes in age-matched BB/Wor DR rats. Although the non-BB rats developed serologic titers to

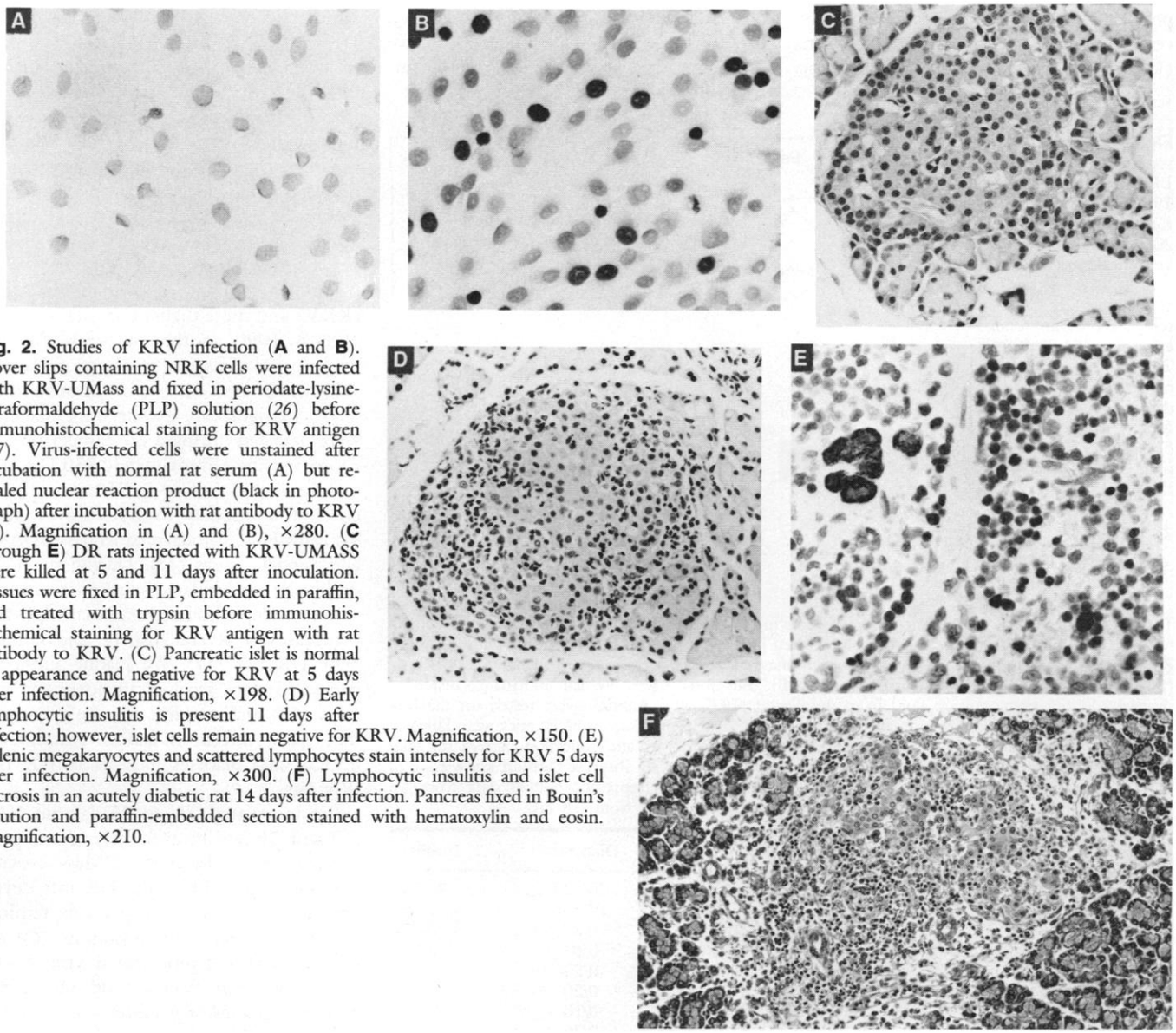

Fig. 2. Studies of KRV infection (**A** and **B**). Cover slips containing NRK cells were infected with KRV-UMass and fixed in periodate-lysine-paraformaldehyde (PLP) solution (*26*) before immunohistochemical staining for KRV antigen (*27*). Virus-infected cells were unstained after incubation with normal rat serum (A) but revealed nuclear reaction product (black in photograph) after incubation with rat antibody to KRV (B). Magnification in (A) and (B), ×280. (**C** through **E**) DR rats injected with KRV-UMASS were killed at 5 and 11 days after inoculation. Tissues were fixed in PLP, embedded in paraffin, and treated with trypsin before immunohistochemical staining for KRV antigen with rat antibody to KRV. (C) Pancreatic islet is normal in appearance and negative for KRV at 5 days after infection. Magnification, ×198. (D) Early lymphocytic insulitis is present 11 days after infection; however, islet cells remain negative for KRV. Magnification, ×150. (E) Splenic megakaryocytes and scattered lymphocytes stain intensely for KRV 5 days after infection. Magnification, ×300. (**F**) Lymphocytic insulitis and islet cell necrosis in an acutely diabetic rat 14 days after infection. Pancreas fixed in Bouin's solution and paraffin-embedded section stained with hematoxylin and eosin. Magnification, ×210.

KRV (*12*), none became diabetic or showed evidence of lymphocytic insulitis. To examine the viral specificity of KRV-induced diabetes, we inoculated DR rats with SDAV (*19*), which was also endemic in our colonies during the episodes of spontaneous DR diabetes (Fig. 1). SDAV-infected animals neither developed diabetes nor insulitis (Table 1). To eliminate the possibility that in vitro–induced cytokines were responsible for β cell injury and diabetes, we purified a KRV-UMass stock away from its putative cytokine-containing culture fluid by ultracentrifugation of the virus through a 50% Renografin (Squibb Diagnostic, New Brunswick, New Jersey) cushion. Forty-seven percent (7/15) of DR rats inoculated with this virus had diabetes or insulitis.

DR rats inoculated with KRV were killed at intervals for serum interferon (IFN) assays (*20*), viral titrations from blood and tissues, and immunocytochemical identification of viral antigen. Serum IFN levels were low and only detectable at 5 days after infection [65 ± 2.5 units/ml (mean ± SEM; n = 5)]. IFN levels at 3, 7, and 10 days after infection were <10 units/ml. KRV plaque-forming units (pfu) were isolated from spleen, liver, and pancreas at all time periods, with the highest titers on days 3 and 5 after infection (Table 2). Blood pfu were relatively low ($<10^3$ pfu/ml) at all time periods, indicating that the organ titers were not due to contaminating blood. KRV antigen was identified in hepatocytes and numerous spleen cells, including lymphocytes and megakaryocytes. KRV antigen was occasionally localized in pancreatic interstitial cell nuclei, but exocrine and endocrine epithelial cells were KRV^- at all times, and there was no evidence of β cell cytolysis before the onset of insulitis (Fig. 2).

Because we reported that immune-suppressive measures induce diabetes in DR rats exposed to environmental pathogens (*13*), we analyzed peripheral blood and lymph node cells of KRV-injected DR rats by flow cytometry for evidence of T cell depletion. When compared with vehicle controls, there were no significant changes in the percentages of peripheral blood $CD4^+$ and $CD8^+$ T cells or natural killer cells at 3, 6, and 10 days after infection or in diabetic rats. $RT6.1^+$ lymph node cells were also unchanged (*21*).

KRV is a common parvovirus that has been isolated from colonies of clinically healthy laboratory rats, as well as feral ones. Adventitious infections of laboratory

rodents with KRV and other rodent parvoviruses are widespread because they are highly stable, small (15- to 28-nm diameter), nonenveloped, single-stranded DNA viruses. They are difficult to eliminate from animal colonies. Infections of immunocompetent animals are generally asymptomatic unless large doses of concentrated virus are given parenterally to perinatal rats and mice or to hamsters that lack indigenous parvoviruses. Serologic evidence of parvovirus infection is also widespread in humans; a report has suggested an association of a parvovirus with rheumatoid arthritis (*22*).

The relation between KRV infection and diabetes in BB/Wor DR rats is based on several lines of evidence. Plaque-purified KRV-UMass, as well as NCI-KRV, reproducibly induced destructive lymphocytic insulitis and diabetes in VAF DR rats. SDAV- and vehicle-inoculated and uninoculated DR rats developed neither insulitis nor diabetes. Inoculated rats uniformly seroconverted to KRV and rarely (<2%) had antibodies to other rodent viruses, indicating that the inocula contained only KRV and that other infections were rare. Finally, KRV was reisolated from experimentally infected animals with lymphocytic insulitis.

Whereas KRV-infected DR rats showed high titers of infectious virus in spleen, liver, and pancreas and viral antigen was localized immunocytochemically in splenocytes and hepatocytes, pancreatic islet and exocrine cells were viral antigen–negative. Furthermore, islet cell necrosis was not evident before the appearance of insulitis. It is therefore unlikely that diabetes resulted from direct lytic infection of the pancreatic β cells. KRV did not induce insulitis or diabetes in non-BB rats, including strains that share the ($RT1^{u/u}$) MHC of BB/Wor rats. These results suggest that genetic factors unique to the BB/Wor rat are essential for this disease.

We have suggested that environmental viral pathogens enhance or inhibit the process of islet cell destruction, depending on the immunologic milieu of the host (*10*). The failure of KRV to accelerate diabetes among DP rats is consistent with the observation that eliminating viral pathogens increased the tempo and frequency of spontaneous diabetes among DP rats. Furthermore, the evidence that KRV induced diabetes in DP rats after they had been reconstituted with DR spleen cells suggests that parvovirus acts by stimulating DR-autoreactive cells, which are required for β cell destruction. The specificity of the requirement for DR cells is supported by initial studies in which eight DP rats reconstituted with Wistar Furth spleen cells did not become diabetic after intraperitoneal injection of 1.4×10^5 pfu of KRV (*21*). That KRV is capable of inducing diabetes in genetically susceptible animals by a process that does not require direct cytolytic infection of pancreatic β cells raises the intriguing possibility that environmental organisms may induce diabetes in susceptible humans by stimulating genetically programmed effector cells or by disrupting a balanced network of autoreactive and regulatory cells.

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12. Serum samples were assayed at CRL (Wilmington, MA) for antibodies to KRV, SDAV, Toolan's H-1 virus, Sendai virus, reovirus type 3, pneumonia virus of mice (PVM), and *Mycoplasma arthritidis* (which is antigenically related to *Mycoplasma pulmonis*) by the enzyme-linked immunosorbent assay (ELISA) (*23*). We prepared test antigens from virus and mycoplasma seeds shown to be free of extraneous organisms, titrated the antigens to determine potency, and tested them against a panel of rodent homologous, heterologous, and nonimmune antisera to determine purity. Each sample was tested for specific antibodies in adjacent wells (96-well microtiter plate), one coated with antigen and the other with tissue control. Tissue controls for the viral antigens were extracts of the uninfected tissue or cells that we used to propagate the virus. Intensity of color development was read at 405 nm with an ELISA plate reader; a score of 0 to 1 was interpreted as negative, 2 was borderline, and 3 or more was positive unless the tissue control was 2 or more. In the latter instance, the result was considered nonspecific.
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14. Virus was isolated from the bone marrows, lymph nodes, spleens, and pancreases of three spontaneously diabetic DR rats and three irradiated nondiabetic DR rats housed with the putatively infected colony. Monolayers of normal rat kidney (NRK) cells were inoculated with tissue homogenates, cultured in Dulbecco's medium, and monitored for virus-induced cytopathic effect (CPE). At 7 days after inoculation, 6/15 wells had CPE distinct from the toxic effects of the organ homogenates. A second series of wells was inoculated and five groups developed CPE without background toxicity. The CPE resembled that induced by a seed stock of NCI KRV (NCI T9530000) obtained from CRL. Syncytial CPE characteristic of paramyxovirus or corona virus were not noted. Positive culture fluids were passed through 0.2-μm filters and produced CPE on subsequent passages.
15. The pancreas isolate was selected for analysis. Cover slips containing infected NRK cells were fixed in an ether-alcohol mixture and stained with rat antiserum to KRV (CRL-S223), followed by fluorescein isothiocyanate–conjugated goat antibody to rat immunoglobulin G (Accurate Chemical). CRL-S223 antiserum was obtained from VAF rats that were enzootically infected with KRV but not other murine viruses. We evaluated antiserum specificity by testing for antibodies to *M. arthritidis*, as well as Sendai virus, PVM, SDAV, KRV, Toolan's H-1 virus, and reovirus type 3. The specificity of KRV antibodies was also confirmed by the ELISA and hemagglutination inhibition tests. Antibodies to other agents were not detected. NRK cells infected with the pancreatic isolate or the defined NCI KRV had nuclear fluorescence, whereas uninfected cells were negative. The isolate was assayed by plaque formation on monolayers of NRK cells overlayed with 2× medium 199 mixed with 1% agarose. The isolate and the NCI KRV induced 0.5-cm plaques of similar morphology. The pancreatic isolate was plaque purified three times, filtered (0.2-μm filter), and used as the virus stock (KRV-UMass) for the described experiments.
16. BB/Wor DR and DP rats were obtained from the research and National Institutes of Health contract colonies maintained at the UMass Medical Center. Congenic PVG-R^8 ($RT1^{a/u}$) and PVG-R^{23} ($RT1^{u/a}$) rats were also bred at UMass. Animals were VAF as determined by quarterly viral antibody screens (*12*). VAF PVG ($RT1^{c/c}$) rats were purchased from Bantin and Kingman, Wistar Furth ($RT1^{u/u}$) rats from the NCI, and Long Evans ($RT1^{u/u}$) rats from CRL. Inoculated animals were housed in a biocontainment suite where they were tested for diabetes three times per week until they were 50 days of age. Diabetics [defined as 4+ glycosuria by TesTape (Eli Lilly, Indianapolis, IN) and a blood glucose of greater than 14 mmol of glucose per deciliter of blood] were killed at detection by intraperitoneal administration of pentobarbital, and nondiabetics were killed at 50 days for morphologic examination of pancreatic islets and collection of serum for viral serology.
17. DP rats at 20 and 21 days were reconstituted with concanavalin A–stimulated DR splenocytes, prepared as reported (*24, 25*). Cells equivalent to one spleen were injected intravenously or intraperitoneally 4 to 7 days before virus inoculation. KRV-injected DP rats were tested for diabetes until they were 50 days of age. Because diabetes occurs spontaneously in about 0.5% of VAF DP rats <50 days old (*10*), the onset of diabetes before 50 days was considered the result of experimental manipulation.
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