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Rotavirus Infection Accelerates Type 1 Diabetes in Mice with Established Insulitis▽

Kate L. Graham,[1]†‡ Natalie Sanders,[1]†‡ Yan Tan,[1]§ Janette Allison,[1,2] Thomas W. H. Kay,[2] and Barbara S. Coulson[1]*

Department of Microbiology and Immunology, The University of Melbourne, Victoria 3010, Australia,[1] and St. Vincent's Institute, Fitzroy, Victoria 3065, Australia[2]

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Infection modulates type 1 diabetes, a common autoimmune disease characterized by the destruction of insulin-producing islet β cells in the pancreas. Childhood rotavirus infections have been associated with exacerbations in islet autoimmunity. Nonobese diabetic (NOD) mice develop lymphocytic islet infiltration (insulitis) and then clinical diabetes, whereas NOD8.3 TCR mice, transgenic for a T-cell receptor (TCR) specific for an important islet autoantigen, show more rapid diabetes onset. Oral infection of infant NOD mice with the monkey rotavirus strain RRV delays diabetes development. Here, the effect of RRV infection on diabetes development once insulitis is established was determined. NOD and NOD8.3 TCR mice were inoculated with RRV aged ≥12 and 5 weeks, respectively. Diabetes onset was significantly accelerated in both models ($P < 0.024$), although RRV infection was asymptomatic and confined to the intestine. The degree of diabetes acceleration was related to the serum antibody titer to RRV. RRV-infected NOD mice showed a possible trend toward increased insulitis development. Infected males showed increased $CD8^+$ T-cell proportions in islets. Levels of β-cell major histocompatibility complex class I expression and islet tumor necrosis factor alpha mRNA were elevated in at least one model. NOD mouse exposure to mouse rotavirus in a natural experiment also accelerated diabetes. Thus, rotavirus infection after β-cell autoimmunity is established affects insulitis and exacerbates diabetes. A possible mechanism involves increased exposure of β cells to immune recognition and activation of autoreactive T cells by proinflammatory cytokines. The timing of infection relative to mouse age and degree of insulitis determines whether diabetes onset is delayed, unaltered, or accelerated.

Type 1 diabetes results from an autoimmune process in which pancreatic β cells are selectively destroyed. An islet lymphoid infiltrate develops that is described as "insulitis" (49). Virus infections are proposed to play a role in type 1 diabetes development through β-cell cytolysis or loss of self-tolerance following pancreatic infection, bystander activation of T cells, and molecular mimicry between β cell autoantigens and viral epitopes (19, 50, 53).

Rotaviruses are the major agents of severe acute gastroenteritis in children and have been implicated in exacerbation of type 1 diabetes development (26). Antibody seroconversion to rotavirus in Australian children was associated with increases in autoantibodies to glutamic acid decarboxylase (GAD) and insuloma-associated protein 2 tyrosine phosphatase (IA-2). Amino acid sequence similarity between rotavirus protein VP7 and T-cell epitopes in human GAD and IA-2 led to the suggestion of T-cell molecular mimicry as a possible mechanism (26, 27). Although later studies in Finnish children did not confirm the association between rotavirus infection and islet autoimmunity (6, 34), increased antibody responses to dietary bovine insulin were noted after rotavirus infection (35). Additional findings that might support links between rotavirus infection and other autoimmunity-related diseases also have been reported (33, 47, 57, 58).

The nonobese diabetic (NOD) mouse spontaneously develops a form of autoimmune diabetes similar to human type 1 diabetes (3, 46). Most mice show severe insulitis by 10 weeks of age. By 30 weeks of age the diabetes incidence typically reaches 60 to 80% in NOD females and 10 to 20% in NOD males. NOD diabetes mainly depends on $CD4^+$ and $CD8^+$ T cells, and most cells in the insulitic lesion are $CD4^+$ T cells. Autoreactive T cells are primed in the draining pancreatic lymph node(s) (PLN) and then migrate to the islets (20, 24, 29). Like humans, NOD mice produce autoantibodies and T cells to GAD and insulin. In addition, $CD8^+$ T cells directed to the islet-specific glucose 6-phosphatase catalytic subunit-related protein (IGRP) are an important component of islet-infiltrating T cells in prediabetic NOD mice (2, 15, 31, 41). Circulating IGRP-reactive T-cell numbers predict diabetes in NOD mice and new-onset patients (36, 52). Expression of the IGRP-specific T-cell receptor (TCR) in NOD mice (NOD8.3 TCR) led to 8.3 TCR expression on >90% of islet-infiltrating T cells and a high diabetes incidence with rapid onset (54, 55). NOD8.3 TCR mice provide a simplified and rapid mouse model of spontaneous diabetes and a useful tool to study the role of $CD8^+$ T cells.

Murine rotaviruses and the rhesus monkey rotavirus strain RRV induce diarrhea in infant mice and infect intestinal cells

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Gate 11, Royal Parade, The University of Melbourne, Melbourne, Victoria 3010, Australia. Phone: 61 3 8344 8823. Fax: 61 3 9347 1540. E-mail: barbarac@unimelb.edu.au.

† K.L.G. and N.S. contributed equally to this study.

‡ Present address: St. Vincent's Institute, Fitzroy, Victoria 3065, Australia.

§ Present address: Department of Dentistry, The University of Melbourne, Victoria 3010, Australia.

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without causing disease in adults, although the doses required differ by several logs (8, 38, 56). Oral RRV infection of infant NOD mice causes gastroenteritis and delays diabetes onset, whereas infection in young adult NOD mice without established insulitis is asymptomatic and diabetes is unaffected (21). RRV infection in infant or young adult NOD mice does not initiate insulitis (21). Infectious RRV spreads to the pancreas in infant NOD mice, with viral antigen localized in macrophages outside islets. Although RRV replicates in islets and pancreatic cells isolated from young adult NOD mice, infectious virus is not detectable in the pancreases of orally inoculated mice of this age (11, 21).

The effect of RRV rotavirus infection on insulitis and diabetes development in older prediabetic NOD and NOD8.3 TCR mice with established insulitis was examined in the present study. RRV is shown here to accelerate diabetes onset and incidence in older prediabetic mice in the absence of detectable extraintestinal spread and pancreatic infection. NOD mice exposed to mouse rotavirus in a natural experiment also showed increased diabetes development. These data provide the first evidence that rotavirus infection can accelerate diabetes development in an animal model.

MATERIALS AND METHODS

Mice. NOD and BALB/c mice were obtained from the Animal Resources Centre (Canning Vale, Western Australia, Australia). NOD8.3 TCR mice, expressing the TCRαβ rearrangements of the H-2K^d-restricted, islet β-cell-reactive CD8$^+$ T-cell clone NY8.3 on a NOD genetic background (54), were provided by P. Santamaria (University of Calgary, Calgary, Alberta, Canada). Mice were bred and housed in the animal facility of the Department of Microbiology and Immunology at the University of Melbourne in isolators under specific-pathogen-free conditions as described previously (21). Offspring of transgenic mice were screened for the transgene by PCR analysis of tail-tip DNA. All procedures were conducted in accordance with protocols approved by the Animal Ethics Committee of The University of Melbourne.

Mouse inoculation with RRV. Monkey rotavirus RRV (serotype P5B[3], G3) was cultivated in MA104 cells, purified by glycerol gradient ultracentrifugation, and titrated for infectivity as described previously (23, 25). Oral inoculation was used to mimic the natural route of infection, as is often done in studies of rotavirus infection in rodents (12, 18, 37). Inoculation procedures were similar to those used previously in our laboratory for NOD mice (21). In brief, after administration of $NaHCO_3$ solution to reduce stomach acidity, mice were inoculated by gavage with 0.2 ml of 50 mM Tris-HCl buffer (pH 7.4), containing 150 mM NaCl and 5 mM $CaCl_2$ (TSC) as a virus diluent control, 1.8×10^6 fluorescent cell-forming units (FCFU) of RRV in TSC, or an extract of mock-infected MA104 cells that had been harvested by freeze-thawing and clarified by low-speed centrifugation as a control for possible cell component contamination of purified RRV. This cell extract was diluted to the same degree as the RRV inoculum. In some experiments (as indicated), mice were given 10^7 FCFU of RRV in TSC. Female and male NOD mice were inoculated at 12 and 15 weeks of age, respectively (unless otherwise indicated) to be confident of their advanced insulitis but avoid infection when diabetes could occur spontaneously. Similarly, NOD8.3 TCR mice were inoculated aged 5 weeks, 1 week before naive mice first begin to develop diabetes. Diarrhea was defined as described previously (21), and its presence was evaluated daily for 8 days after inoculation.

Mouse inoculation with mouse rotavirus. Five-day-old BALB/c mice housed with their rotavirus-seronegative dams in an isolation room in the animal facility were inoculated by oral gavage as described previously (21) with 30 μl of TSC (controls; $n = 42$) or clarified stool homogenate (10% [wt/vol] in TSC) from a diarrheic mouse ($n = 42$). In an enzyme immunoassay (EIA) using rabbit antiserum to RRV as capture antibody and monoclonal antibody RVA as a detector antibody (21), this stool extract showed a mean specific optical density at 450 nm (OD_{450}) ± the standard deviation of 1.196 ± 0.066, indicating that a high level of rotavirus antigen was present. According to the previously proposed nomenclature scheme for mouse rotaviruses, this virus is designated as the agent of epizootic diarrhea of infant mice (EDIM)-Melbourne, abbreviated as EM here (8). Inoculated mice were monitored for diarrhea as described above.

Detection of rotavirus in organs and samples from rotavirus-infected mice. For analysis of RRV-infected NOD and NOD8.3 TCR mice, the small intestine, pancreas, liver, spleen, serum, and blood cells were obtained from each animal at days 3 to 8 after infection ($n = 4$ per day). Stools were collected (one pellet per mouse) at days 2 ($n = 20$), 3 ($n = 20$), 4 ($n = 20$), 5 ($n = 16$), 6 ($n = 12$), 7 ($n = 8$), and 8 ($n = 4$) after infection. The procedures for collection and processing of these tissues and stools have been described previously (21). Infectious RRV was detected by culture amplification of virus, followed by assay of rotavirus antigen in cultures by capture EIA, and stools and pancreases were examined for rotavirus antigen by EIA prior to culture, as described previously (21).

The pancreas, liver, spleen, serum, and blood cells were collected from each BALB/c mouse infected with mouse rotavirus EM and control BALB/c mice at days 2 to 7 after infection ($n = 5$ per day) and processed as described previously (21). Intestinal cells were obtained by using established methods (14, 59). In brief, the small intestine was placed in Hanks balanced salt solution containing 5% (vol/vol) fetal bovine serum (CSL, Ltd., Melbourne, Australia) and 1 mM dithiothreitol (Sigma). Intestines were cut open lengthwise and then into 1-cm pieces. Cells (including enterocytes) were detached by using an orbital shaker at 37°C for 40 min, filtered through a 70-μm-pore-size cell strainer, and pelleted by centrifugation at $450 \times g$ for 5 min. Since EM was not culture adapted, rotavirus antigen in organs and samples (except the small intestine) from infected BALB/c mice was detected by capture EIA without culture amplification. The proportion of detached intestinal cells expressing rotavirus antigen was determined by flow cytometric analysis of methanol-fixed cells, as described previously (22).

Assay for rotavirus antibodies. Titers of rotavirus antibodies were determined in the sera from all mice by EIA using RRV antigen, as previously described (21). All mice were negative for serum antibodies to RRV prior to experimentation. Seroconversion was defined as a fourfold increase in antibody titer.

Glucose homeostasis and diabetes monitoring. Glycosuria was monitored by using Diastix reagent strips at 1 day prior to RRV or control inoculation and at days 3, 5, 7, 9, and 11 after infection. In the weekly screening after inoculation, urine testing was alternated with measurement of blood levels by using an Accu-Check Advantage II blood glucose meter and strips. Blood glucose levels were determined immediately in glycosuric mice. As described previously, consecutive blood glucose levels of >240 mg/dl on two occasions 2 to 3 days apart were considered to indicate diabetes development (21).

Histology. Pancreases were fixed in Bouin's solution and embedded in paraffin. Sections (5 μm) were cut 200 μm apart at four levels and stained with hematoxylin and eosin as previously described (21). For each mouse a quantitative insulitis score was determined by using an adaptation of a previously described method (43). All islets present in each level of the pancreas (at least 20 per mouse) were scored as 0 (no islet infiltrate), 1 (insulitis visible around the outer edge of the islet), 2 (intra-islet infiltration into <30% of total islet area), 3 (intra-islet infiltration into 30 to <70% of total islet area), or 4 (infiltration into 70 to 100% of the islet).

Isolation and flow cytometric analysis of cells from islets and PLN. Purified islets were obtained from pancreases by bile duct cannulation, collagenase digestion, and density gradient separation, as described previously (32). Cells released from islets with trypsin-EDTA were allowed to recover in culture medium for 0.5 to 1.5 h before analysis, as described previously (13). PLN were dissected from harvested pancreases, mechanically disrupted by using glass slides, filtered through nylon mesh, and washed. Single-cell suspensions from islets and PLN were labeled for T- and B-cell analysis with combinations of monoclonal antibodies specific to mouse antigens (BD Biosciences). Cells were labeled with fluorescein isothiocyanate-conjugated CD4 (GK1.5), phycoerythrin-conjugated CD45R/B220 (RA3-6B2), and CyC-conjugated CD8a (53-6.7). In addition, islet cells were labeled with allophycocyanin-conjugated anti-CD45 (30-F11) to isolate lymphocytes from other cell types. For detection of major histocompatibility complex class I (MHC-I), islet cells were labeled with biotinylated anti-H-2K^d and allophycocyanin-conjugated anti-CD45, followed by phycoerythrin-conjugated streptavidin. Propidium iodide at 50 μg/ml was included at the final step to allow the exclusion of dead cells. Cell data were acquired on a FACScalibur flow cytometer and analyzed with CellQuest Pro software v5.2 (BD Biosciences). Forward and side scatter gates for lymphocytes were set on CD45$^+$ cells from islets and on all PLN cells. These lymphocytes were then analyzed for CD4, CD8, and B220.

Islet expression of TNF-α mRNA. Total RNA was extracted from purified islets by using TRIzol reagent (Invitrogen). Total RNA was reverse transcribed by using random primers. Real-time PCR was conducted using Assays-on-Demand kits (Applied Biosystems) for tumor necrosis factor alpha (TNF-α) and β actin as a reference gene, in a Corbett Research Rotor-Gene 3000 sequence detector.

Statistical analysis. The Student paired *t* test, Mann-Whitney test, and one-way analysis of variance (ANOVA) were used as appropriate. Other tests were used as indicated. Diabetes curves were evaluated by Kaplan-Meier life-table analysis and the log-rank test for trend.

RESULTS

Older NOD and NOD8.3 TCR mice infected with RRV showed asymptomatic infection, limited intestinal replication, and no viremia or extraintestinal spread. In order to examine virological parameters of rotavirus infection in insulitic mice, NOD mice aged 12 weeks (females) or 15 weeks (males) and NOD8.3 TCR mice aged 5 weeks were infected by oral gavage with RRV rotavirus. All RRV-inoculated mice seroconverted to homologous rotavirus by 2 weeks after infection, whereas all diluent-inoculated mice showed negative antibody titers to RRV of <1:50 (see Fig. 3; also data not shown). No RRV-infected or control mice showed diarrhea. On day 4 after infection, 5% (1/20) of stools from both male and female NOD mice contained infectious RRV, and a stool from another female mouse contained rotavirus antigen but not infectious virus. On day 7 after infection, 12% (1/8) of stools from females also contained RRV antigen. Males did not excrete any detectable rotavirus antigen at any time after infection. Overall, 23% of female NOD mice and 5% of males excreted detectable infectious RRV and/or rotavirus antigen. On days 2 to 4 after infection of approximately equal numbers of female and male NOD8.3 TCR mice, stools from 23% of females and 18% of males contained infectious RRV, and 25% (1/4) of the small intestines collected on day 4 contained infectious RRV. Overall, RRV was found intestinally in 45% of NOD8.3 TCR mice. The altered T-cell repertoire of NOD8.3 TCR mice might have contributed to their increased rate of intestinal RRV detection over NOD mice, through a reduced ability to control RRV replication. The stool and intestinal extracts generally contained low levels of infectious RRV, showing OD_{460} values for RRV antigen by EIA after culture amplification of ≤ 0.25, with one exception ($OD_{460} = 1.1$). In our experience, samples with OD_{460} values of ≤ 0.25 contain levels of infectious RRV below the detection limit for direct titration in permissive cells (4×10^2 FCFU/ml), so titration was not attempted (21). Infectious RRV was not detected in the pancreases, livers, spleens, sera, or blood cells of these NOD and NOD8.3 TCR mice by culture amplification and then EIA. This method was successfully used in our laboratory by the same experimenters in the same year to detect infectious rotavirus at these sites in RRV-infected infant NOD mice (21). In addition, RRV antigen was not detected in the pancreas by EIA.

RRV infection of female and male NOD mice with established pancreatic insulitis accelerated diabetes development. To determine whether rotavirus infection modulates the timing and incidence of spontaneous diabetes onset, groups of 12- to 15-week-old NOD mice were orally inoculated with RRV, virus diluent, or cell extract and then monitored for glycosuria and hyperglycemia (Fig. 1). These studies overlapped in time and location with closely related experiments undertaken by our group, which showed that oral RRV inoculation of NOD mice delays diabetes onset in infants and has little effect in adults aged 4 to 6 weeks (21). Mice inoculated with cell extract were included to control for the effect of any contamination of purified RRV with immunostimulatory components from cells used to propagate RRV.

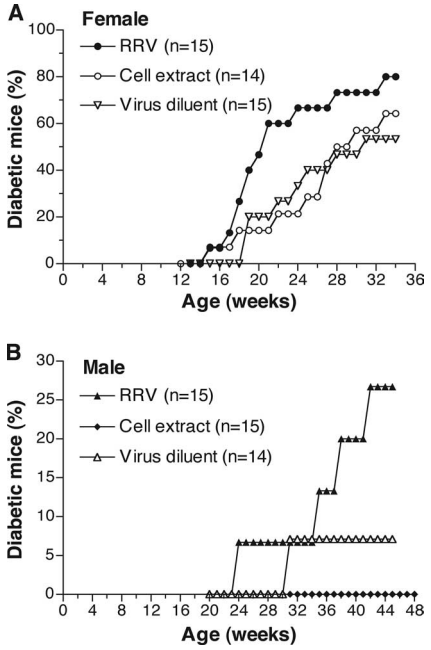

FIG. 1. Diabetes development was accelerated by RRV infection of female (A) and male (B) NOD mice with established insulitis. Mice were inoculated orally at 12 weeks (female) or 15 weeks (male) of age with RRV, virus diluent, or cell extract and then monitored for diabetes until 34 weeks (female) or 45 weeks (male) of age. The data provided are from a single experiment that is representative of two independent experiments, each performed with similar numbers of mice.

NOD mice did not show glucosuria in the 2 weeks after RRV infection or control inoculations. However, hyperglycemia and diabetes began to develop at 3 weeks (females) and 9 weeks (males) after RRV infection, 4 weeks (females) and 7 weeks (males) earlier than mice inoculated with virus diluent (Fig. 1).

Two female NOD mice inoculated with cell extract developed diabetes 1 week and 4 weeks earlier than any virus diluent-inoculated females (Fig. 1A). However, there was no significant difference between the diabetes curves of females inoculated with virus diluent or cell extract ($P = 0.73$), indicating that any MA104 cell components in the purified RRV preparation would not materially affect diabetes development. A significant acceleration in diabetes development was observed from the diabetes curves of RRV-infected females compared to females fed virus diluent ($P = 0.023$) or cell extract ($P = 0.019$). The proportion of females with diabetes at 21 weeks of age increased from 14% (2/14) in those fed cell extract to 60% (9/15) after RRV infection ($P = 0.021$). However, by 34 weeks of age the diabetes proportions in control and RRV-infected mice were similar ($0.24 < P < 0.43$).

The diabetes curves of cell extract- and RRV-inoculated males showed no significant difference from those of diluent-fed males (Fig. 1B; $P = 0.30$ and $P = 0.18$, respectively). However, by survival analysis RRV-infected male mice showed a significant acceleration in diabetes development compared to mice fed cell extract ($P = 0.035$). Compared to diluent- and

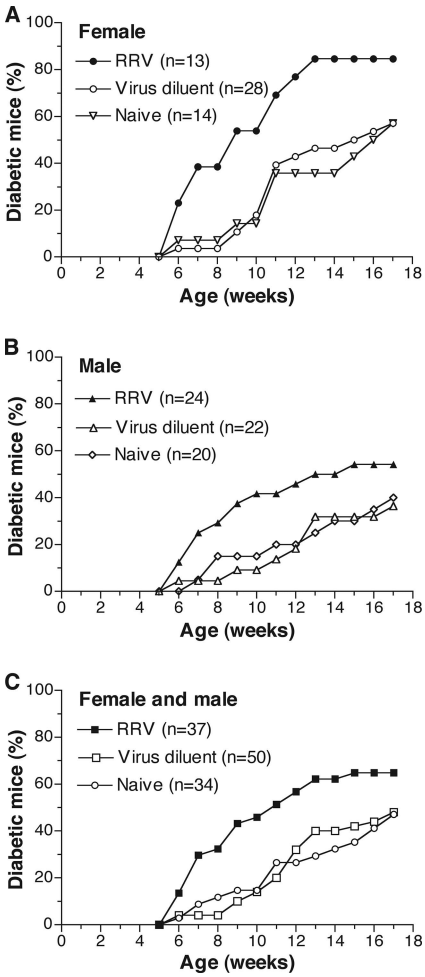

FIG. 2. Diabetes development was accelerated by RRV infection of female (A), male (B), or female and male (C) NOD8.3 TCR mice with established insulitis. Mice were orally inoculated at age 5 weeks with RRV or virus diluent and monitored for diabetes until 17 weeks of age. The data were compiled from four independent experiments performed over a 1-year period, each of which showed similar patterns of diabetes development. The naive NOD8.3 TCR mouse curves represent spontaneous diabetes development in the animal facility during this time.

extract-fed mice, the proportion of diabetic males at 45 weeks of age increased >4-fold after RRV infection, from 7% (1/14) and 0% (0/15), respectively, to 27% (4/15), although this was not statistically significant ($P = 0.33$ and $P = 0.09$, respectively).

RRV infection of female and male NOD8.3 TCR mice with established pancreatic insulitis accelerated diabetes development. The effect of RRV infection on the timing and incidence of diabetes was examined in NOD 8.3 TCR mice, which spontaneously develop diabetes more rapidly than NOD mice. As shown in Fig. 2A, the diabetes survival curves in diluent-inoculated and naive female NOD8.3 TCR mice were indistinguishable ($P = 0.45$). Compared to diluent-inoculated and naive females, RRV-infected females showed a highly significant acceleration in timing of diabetes onset and increased diabetes incidence ($P = 0.0052$ and $P = 0.0072$, respectively). The increased diabetes incidence was evident 1 week after infection and maintained to 17 weeks of age (Table 1). These findings indicate that the kinetics of diabetes onset was accelerated by RRV infection.

Male NOD8.3 TCR mice showed a lower diabetes incidence than females, as expected from previous studies and the NOD genetic background of these mice (54). The diabetes survival curves in diluent-inoculated and naive male NOD8.3 TCR mice were indistinguishable (Fig. 2B; $P = 0.85$). RRV infection significantly accelerated diabetes onset and incidence in males compared to diluent inoculation ($P = 0.038$), and a trend for infected males to show accelerated diabetes over naive males was evident ($P = 0.06$). RRV-infected and control mice showed similar diabetes incidences at 17 weeks of age (Table 1).

Relatively high proportions of naive female (57%; 16/28) and male (36%; 8/22) NOD8.3 TCR mice became diabetic by 17 weeks of age, and the sexes showed similar overall patterns of diabetes development following diluent and rotavirus inoculation (Fig. 2A and B). This allowed combination of the sexes for overall analysis, in contrast to NOD mice (Fig. 2C). The rates of diabetes observed irrespective of sex in diluent-inoculated and naive NOD8.3 TCR mice were indistinguishable ($P = 0.16$). However, diabetes onset was more rapid in RRV-infected mice, particularly in the first 3 weeks after infection (Table 1). After this time the slopes of the diabetes curves of RRV-inoculated, diluent-inoculated, and naive mice were similar, again showing that the diabetes acceleration induced by RRV manifested predominantly in the first 3 weeks. Overall, RRV infection significantly accelerated the onset of diabetes over diluent-inoculated and naive mice ($P = 0.008$ by survival analysis). The proportions of diabetic mice at 17 weeks of age did not differ between test and control mice (Table 1). However, a subset of these mice (13 females and 19 males) was monitored until 19 to 20 weeks of age. The diabetes incidence in this subset increased from 47% (7/15) in diluent-inoculated

TABLE 1. Effects of RRV infection on the timing of diabetes onset in NOD8.3 TCR mice

Time (wk) after inoculation	Proportion (%) of diabetic mice after the indicated inoculation								
	Female			Male			Total		
	RRV	Diluent	None	RRV	Diluent	None	RRV	Diluent	None
1	3/13 (23)	1/28 (3)	1/14 (7)	2/24 (8)	1/22 (4)	0/20 (0)	5/37 (14)	2/50 (4)	1/34 (3)
3	5/13 (39)	1/28 (3)	1/14 (7)	7/24 (29)	1/22 (4)	3/20 (15)	12/37 (32)[a]	2/50 (4)	4/34 (12)
12	11/13 (85)[b]	16/28 (57)	8/14 (57)	13/24 (54)[c]	8/22 (36)	8/20 (40)	24/37 (65)[d]	24/50 (48)	16/34 (47)

[a] $P = 0.0005$ (Fisher exact test).
[b] $P = 0.0035$ (Fisher exact test).
[c] $0.25 < P < 0.38$ (Fisher exact test).
[d] $0.059 < P < 0.066$ (chi-square test).

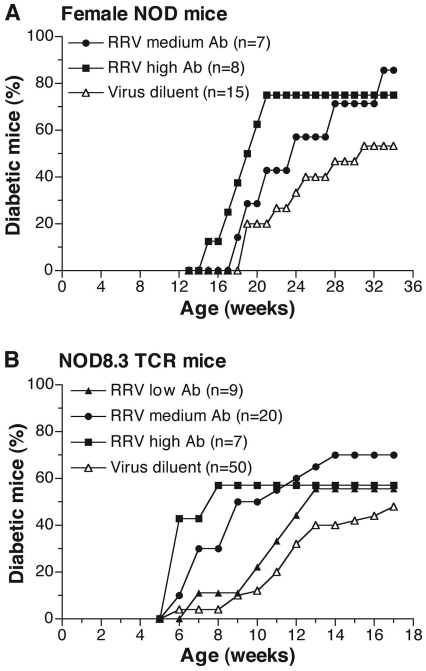

FIG. 3. Diabetes acceleration was of greater extent in mice showing higher titers of anti-rotavirus antibody (Ab) in serum. The diabetes curves for RRV-infected female NOD mice from Fig. 1 (A) and NOD8.3 TCR mice from Fig. 2 (B) were stratified into mice with low (1:100 to 1:400), medium (1:800 to 1:3,200), and high (1:6,400 to 1:25,600) serum antibody titers to RRV at 2 weeks after infection. Serum from one NOD8.3 TCR mouse was unavailable for antibody testing, so the results from that mouse were excluded.

mice to 88% (15/17) in RRV-infected mice (Fisher exact test, $P = 0.021$).

Relation between serum antibody titers to RRV and degree of diabetes acceleration. Diabetic and nondiabetic mice inoculated with RRV showed similar geometric mean titers of serum anti-rotavirus antibodies (NOD, 1:4,300 and 1:3,200, respectively; NOD8.3 TCR, 1:1,200 and 1:1,500, respectively; $P > 0.05$). The reduced NOD8.3 TCR mouse titers probably resulted from their altered T-cell repertoire. Stratification of RRV-infected mice by antibody titer demonstrated that the extent of diabetes acceleration was significantly greater in mice showing higher titers of anti-rotavirus antibody in serum (Fig. 3; survival analysis and log-rank test for trend, $P = 0.023$ and $P = 0.0074$ for NOD, respectively, and $P = 0.042$ and $P = 0.0059$ for NOD8.3 TCR, respectively). High-responding NOD and NOD8.3 TCR mice developed diabetes significantly earlier than diluent-inoculated mice (Table 2; $0.017 < P < 0.028$). NOD mouse inoculation with a fivefold-higher dose of RRV (1.0×10^7 FCFU) did not materially affect the diabetes curves or seroconversion antibody titers obtained (data not shown).

TABLE 2. Relation between serum antibody titers to RRV and mouse age at diabetes onset

Mice	Mean age (wk) ± SD at diabetes onset of mice with antibody titers to RRV of:			
	<1:50[a]	1:100–1:400	1:800–1:3,200	1:6,400–1:25,600
Female NOD	24.2 ± 5.1	–[b]	22.9 ± 4.3	19.3 ± 3.2[c]
NOD8.3 TCR	11.5 ± 2.9	10.6 ± 2.3	9.7 ± 3.6	6.5 ± 1.0[d]

[a] Diluent-fed mice.
[b] RRV-infected NOD mice did not show titers in this range.
[c] $P = 0.027$ compared to diluent-fed mice.
[d] $P = 0.018$ compared to diluent-fed mice.

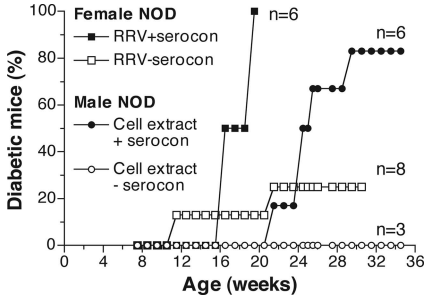

FIG. 4. Effect of exposure to mouse rotavirus EM in a natural experiment on diabetes development in NOD mice. Mice are stratified into those that seroconverted to rotavirus after possible exposure to EM (+serocon) and those that did not seroconvert (−serocon). Females had been inoculated with RRV at 4 weeks of age prior to EM exposure, whereas males had been inoculated at 4 weeks of age with control cell extract but had no previous rotavirus exposure.

Rotavirus infection of NOD mice at 10 to 20 weeks of age in natural experiments. Several older adult NOD mice were inadvertently exposed to EM mouse rotavirus infection when coquarantined with mice imported from a rotavirus-positive facility. Since EM exposure was detected during monitoring of antibodies to RRV by EIA in sera collected fortnightly or monthly, its timing could be determined to within a 2- to 4-week interval. No gastroenteritis was observed in the imported mice or the exposed older adult NOD mice during this period, suggesting EM was of low virulence for older adults. The NOD mice were monitored for diabetes until 35 weeks old.

Most (6/9) of the adult male NOD mice that were exposed to EM seroconverted to rotavirus, aged 13 to 20 weeks, and 5 of 6 (81%) of these EM-exposed mice developed diabetes aged a mean ± the standard deviation of 25 ± 2 weeks (Fig. 4). In contrast, none (0/3) of the EM-exposed male mice that did not seroconvert developed diabetes (Fisher exact test, $P = 0.04$). The male diabetes incidence in this facility is ~10% at 30 weeks (Fig. 1), highlighting the extent of the increased diabetes incidence after rotavirus seroconversion. Females that had seroconverted to rotavirus after oral RRV inoculation at 4 weeks of age also were inadvertently exposed to EM. Many of these females (6/14; 43%) seroconverted to rotavirus a second time at 10 to 14 weeks of age. These mice all developed diabetes, at 18 ± 2 weeks of age (Fig. 4). In contrast, only 2 of 8 (25%) of the RRV-inoculated females that did not seroconvert a second time developed diabetes, at 16 and 25 weeks of age ($P = 0.01$). The diabetes incidence in female NOD mice in this facility was ~60% at 30 weeks (Fig. 1). These findings of diabetes acceleration in highly insulitic NOD mice after EM rotavirus exposure are consistent with the results obtained from the RRV infection experiments described above that were conducted in NOD mice under controlled conditions.

All BALB/c mouse pups inoculated with a stool extract containing high levels of EM antigen showed diarrhea that re-

TABLE 3. Intestinal replication of mouse rotavirus EM in infant BALB/c mice

Time (days) after EM inoculation	Mice showing rotavirus antigen in intestinal cells (%)	Intestinal cells containing rotavirus antigen (mean % ± SEM)
2	100	3.4 ± 0.6
3	100	5.9 ± 0.3
4	100	5.6 ± 0.6
5	40	1.6 ± 0.8
6	20	0.1 ± 0.1
7	0	0.0 ± 0.0

solved without mortality and rotavirus antigen in intestinal cells for 6 days after inoculation. Up to 6% of intestinal cells contained antigen (Table 3). No rotavirus antigen was detected by direct EIA in the pancreas, liver, spleen, serum, or blood cell preparations. These data indicate that EM replicated efficiently in the small intestine without any detectable extraintestinal spread.

Effect of RRV infection on pancreatic insulitis in NOD mice. The ability of RRV infection to accelerate diabetes development in NOD mice suggested that islet insulitis also might be increased after RRV infection. The degree of insulitis was scored at a range of times after RRV infection or virus diluent inoculation of female and male NOD mice (Fig. 5).

As expected due to the ongoing diabetic process, insulitis scores in cell extract-inoculated female mice significantly increased between 13 and 17 weeks of age (Fig. 5A; $P = 0.017$). A similar effect was seen in RRV-infected females (Fig. 5A; $P = 0.039$), indicating that RRV infection did not inhibit ongoing insulitis development. No significant difference in insulitis scores between RRV-infected and cell extract-inoculated females was observed at 13 or 17 weeks ($P = 0.43$ and $P = 0.76$, respectively). However, the upper range of insulitis scores was increased 1 week after RRV infection over cell extract-inoculated mice at the same time (Fig. 5A), suggesting a possible trend for RRV-infected females to show higher scores than control mice. This is consistent with the accelerated diabetes onset in these mice. Insulitis scores at 13 weeks of age also were determined for females previously inoculated with RRV or cell extract at 5 days of age (Fig. 5A). Consistent with our previous data (21), RRV infection at 5 days of age did not significantly alter insulitis scores ($P = 0.39$). There was no significant difference in the degree of insulitis at 13 weeks of age between mice inoculated with the control preparation at 5 days or 12 weeks ($P = 0.61$). However, females previously infected with RRV aged 12 weeks showed significantly elevated insulitis at 13 weeks of age compared to those of the same age that were inoculated with RRV at 5 days of age (Fig. 5A; $P = 0.036$).

No significant differences in scores between RRV-infected and control male NOD mice were evident at 16, 20, or 52 weeks of age (Fig. 5B; $P = 0.51$, $P = 0.49$, and $P = 0.24$, respectively). However, insulitis scores in RRV-infected males increased significantly between 1 and 5 weeks ($P = 0.048$) and 1 and 37 weeks after infection ($P = 0.028$). In contrast, mean insulitis scores in control mice did not alter from 1 to 37 weeks after infection ($0.38 < P < 0.69$). Several mice analyzed at 52 weeks showed insulitis scores similar to those of diabetic males, but mice analyzed earlier generally showed lower scores, as expected.

Overall, RRV infection did not inhibit insulitis development in older female or male NOD mice, and evidence of a possible trend for increased insulitis after RRV infection was found for both sexes.

Analysis of lymphocytes in islets and PLN following RRV infection of NOD mice. To further analyze the insulitis following RRV infection of older NOD mice, the proportions of $CD4^+$ T cells, $CD8^+$ T cells, and B cells infiltrating islets and trafficking through the PLN were examined in female and male NOD mice (Tables 4 and 5). There were no significant differences in the proportions of $CD4^+$ T cells, $CD8^+$ T cells, and B cells in islets or PLN between RRV-infected and cell extract-inoculated female mice ($P > 0.05$). However, RRV infection of male mice significantly increased the proportion of $CD8^+$ T cells in islets at 1 week after infection compared to cell-extract-inoculated mice ($P = 0.039$; Table 4). B-cell proportions in PLN and islets were significantly increased over controls at 3 weeks and 5 weeks after RRV infection of males, respectively ($P = 0.005$ and $P = 0.048$, respectively; Tables 4 and 5). The

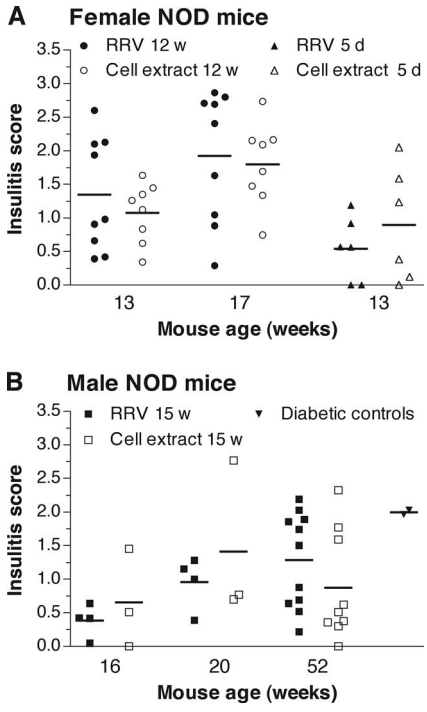

FIG. 5. Effects of RRV infection on insulitis development in NOD mice. (A) The pancreases of female NOD mice inoculated with RRV or virus diluent at 12 weeks of age were scored for insulitis at 1 and 5 weeks after infection, at the ages of 13 and 17 weeks, respectively. These data are compared to the insulitis scores at a similar age (13 weeks) in the pancreases of female NOD mice inoculated by oral gavage at 5 days of age. Nondiabetic mice only were included. (B) Pancreatic insulitis scores at 16, 20, and 52 weeks of age in male NOD mice inoculated with RRV or diluent at 15 weeks of age that had not become diabetic. As positive controls, the pancreas was collected at diabetes onset from two male NOD mice (one RRV inoculated) and analyzed for insulitis. All RRV-infected mice showed serum antibody titers of <1:50 and 1:3,200 at 1 and 5 weeks after infection, respectively. The bars indicate the means.

TABLE 4. Proportions of lymphocyte subsets in islets of NOD mice inoculated with cell extract or RRV

Time (wk) after inoculation[a]	Mean % of lymphocytes ± SEM in islets with the indicated cell surface marker[b]					
	CD4+		CD8+		B220+	
	Cell extract	RRV	Cell extract	RRV	Cell extract	RRV
Females						
1 ($n = 10$)	38.5 ± 2.6	36.6 ± 1.5	17.9 ± 1.1	18.6 ± 1.3	25.0 ± 1.4	26.8 ± 2.2
2 ($n = 8$)	38.2 ± 1.4	38.1 ± 1.9	20.2 ± 0.5	21.3 ± 1.4	23.2 ± 1.5	23.8 ± 1.9
5 ($n = 9$)	44.0 ± 1.6	44.2 ± 2.7	18.1 ± 1.3	20.2 ± 0.9	23.9 ± 2.1	20.6 ± 3.2
8 ($n = 8$)	39.0 ± 2.8	32.8 ± 1.0	18.2 ± 1.3	17.5 ± 1.9	21.3 ± 2.4	27.9 ± 4.4
Males						
1 ($n = 19$)[c]	36.1 ± 2.3	32.3 ± 1.6	**16.1 ± 2.1**	**21.4 ± 1.3**[d]	30.1 ± 2.3	30.2 ± 2.1
3 ($n = 7$)	39.8 ± 1.2	39.5 ± 2.1	14.2 ± 1.3	17.4 ± 1.0	27.3 ± 0.9	28.4 ± 3.1
5 ($n = 7$)	42.6 ± 2.1	37.7 ± 1.5	18.4 ± 0.7	15.4 ± 1.4	**23.6 ± 1.4**	**29.8 ± 2.2**[e]
7 ($n = 6$)	33.6 ± 2.5	36.8 ± 2.2	16.6 ± 1.2	18.4 ± 1.3	31.3 ± 2.3	26.7 ± 2.1
10 ($n = 5$)	31.8 ± 2.0	35.5 ± 2.7	11.2 ± 0.8	13.2 ± 1.0	35.0 ± 3.4	31.6 ± 3.5

[a] n, Number of animals tested.
[b] Lymphocyte subsets showing significant differences in proportions are indicated in boldface.
[c] Except for the cell extract group, where $n = 11$.
[d] $P = 0.039$.
[e] $P = 0.048$.

CD4+/CD8+ T-cell ratios also were determined for these mice (Fig. 6). In the first 8 weeks after RRV infection, CD4+/CD8+ ratios in islets and PLN of female NOD mice were unaltered compared to extract-inoculated mice (Fig. 6; $P > 0.05$). However, CD4+/CD8+ ratios in islets decreased significantly in male NOD mice 1 week after RRV infection compared to extract-fed mice (Fig. 6; $P = 0.003$). Overall, these data indicate that T-lymphocyte proportions in islets and PLN were unaltered in female NOD mice after RRV infection. However, RRV infection in male NOD mice led to increased CD8+ T-cell proportions and reduced CD4+/CD8+ ratios in islets at 1 week after infection.

MHC-I expression on β cells was increased after RRV infection in NOD8.3 TCR mice. Upregulation of MHC-I on β cells has been proposed to be important for triggering progression to diabetes (17). The ability of rotavirus infection to modify MHC-I expression by β cells was analyzed. As shown in Fig. 7, MHC-I levels increased significantly over time in both mock- and RRV-infected NOD8.3 TCR mice (ANOVA post test for linear trend, $P < 0.0003$). RRV infection produced significantly increased MHC-I levels on NOD8.3 TCR β cells at day 10 after infection compared to mock-infected mice on the same day ($P = 0.012$). MHC-I levels in female NOD mice were examined at days 1 to 5, 7, 14, and 35 after mock or RRV infection. In female NOD mice, β cell MHC-I expression increases with age more slowly than NOD8.3 TCR MHC-I (48). Consistent with this, β cell MHC-I levels did not alter significantly over the study period in mock- or RRV-infected mice (data not shown; ANOVA post test for linear trend, $P > 0.05$). MHC-I levels in RRV-infected female NOD mice were similar to those in mock-infected mice on the same day (data not shown; $P > 0.05$). Thus, alteration in MHC-I levels was not detected in the 35 days following RRV infection of female NOD mice.

TABLE 5. Proportions of lymphocyte subsets in PLNs of NOD mice inoculated with cell extract or RRV

Time (wk) after inoculation[a]	Mean % of lymphocytes ± SEM in the PLN with the indicated cell surface marker[b]					
	CD4+		CD8+		B220+	
	Cell extract	RRV	Cell extract	RRV	Cell extract	RRV
Females						
1 ($n = 10$)	40.3 ± 2.3	42.2 ± 2.1	17.1 ± 0.8	17.1 ± 0.8	9.7 ± 1.5	12.1 ± 1.6
2 ($n = 7$)	35.5 ± 1.3	38.3 ± 2.6	15.7 ± 0.8	15.1 ± 0.8	11.2 ± 1.6	7.6 ± 1.1
5 ($n = 9$)	33.8 ± 2.0	37.1 ± 1.3	14.9 ± 1.5	13.4 ± 1.6	12.0 ± 1.4	10.9 ± 1.7
8 ($n = 8$)	38.1 ± 1.4	38.5 ± 2.4	16.9 ± 0.9	17.1 ± 0.7	9.7 ± 1.5	9.8 ± 2.0
Males						
1 ($n = 12$)[c]	35.6 ± 2.5	32.9 ± 2.0	16.1 ± 1.0	14.6 ± 0.7	12.2 ± 2.9	12.1 ± 1.4
3 ($n = 7$)	27.0 ± 2.4	28.6 ± 2.7	18.0 ± 2.9	14.4 ± 1.7	**7.1 ± 0.3**	**11.2 ± 1.0**[d]
5 ($n = 7$)	24.3 ± 2.1	28.4 ± 4.2	13.7 ± 1.0	15.0 ± 2.1	11.8 ± 3.9	9.2 ± 1.7
7 ($n = 6$)	23.4 ± 5.0	29.7 ± 3.8	14.5 ± 2.1	16.6 ± 1.7	10.1 ± 1.5	11.7 ± 1.6
10 ($n = 5$)	36.2 ± 2.2	39.6 ± 1.5	15.9 ± 1.3	18.6 ± 0.3	10.9 ± 1.7	12.0 ± 0.9

[a] n, Number of animals tested.
[b] Lymphocyte subsets showing significant differences in proportions are indicated in boldface.
[c] Except for the cell extract group, where $n = 5$.
[d] $P = 0.0047$ (Student t test with Welch's correction).

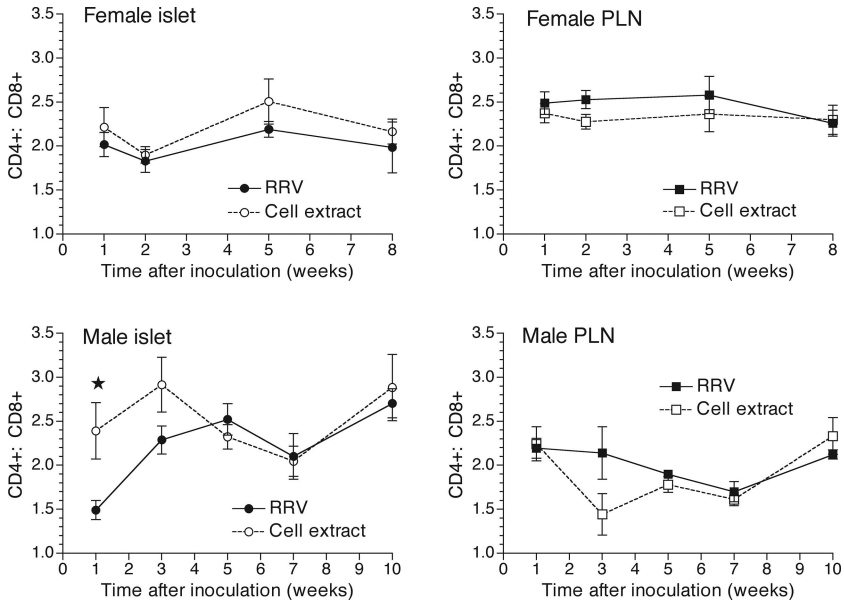

FIG. 6. Effects of RRV infection on ratios of $CD4^+$ to $CD8^+$ T cells in islets and PLN of NOD mice. At each time point, at least 5,000 lymphocyte-sized cells were analyzed for each cell marker (as described in Materials and Methods) from the groups of mice described in Tables 4 and 5 that had not become diabetic. All RRV-inoculated mice seroconverted by 2 weeks after infection. Mice showed a geometric mean serum antibody titer of 1:2,800 at 5 weeks after infection. Error bars represent the standard error of the mean. A significant difference in ratios between RRV-infected and control mice is marked with a star ($P = 0.003$).

Islet TNF-α levels in insulitic mice were increased by RRV infection. Proinflammatory cytokines such as TNF-α are cytotoxic to islets. TNF-α mRNA levels were determined in isolated islets of mock- and RRV-infected mice (Fig. 8). RRV infection significantly increased TNF-α mRNA levels at 10 to 15 days postinfection in NOD8.3 TCR mice ($P = 0.036$), and 7 to 14 days in female NOD mice ($P = 0.0033$). TNF-α mRNA levels were unchanged by RRV infection at other times.

DISCUSSION

Experiments presented here show that RRV rotavirus infection of diabetes-prone mice with established insulitis accelerates diabetes development. Thus, rotavirus can hasten diabetes onset once β-cell autoimmunity is established. This is the first described study in an animal model suggesting a relationship between rotavirus infection and diabetes exacerbation. These findings support the proposed association of rotavirus infection with increased islet autoimmunity in children (26). Taken in conjunction with our previous demonstration that RRV infection in preinsulitic NOD mice can delay diabetes development (21), these new results indicate that the timing of RRV infection in relation to mouse age and degree of insulitis determines whether diabetes onset is accelerated, delayed, or unaltered.

The specificity of the diabetes acceleration for RRV is highlighted by the lack of diabetes modulation by control cell extracts, and the overlap in time and place of execution between the accelerated diabetes curves after RRV infection in older adult mice described here and those showing diabetes delay in

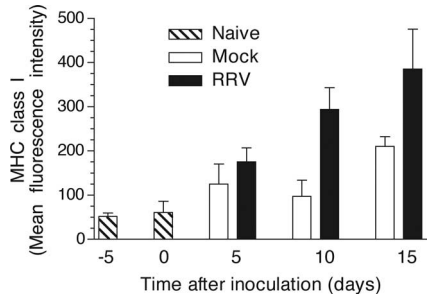

FIG. 7. Expression of MHC-I on β cells of NOD8.3 TCR mice after RRV infection. Viable, CD45-negative islet cells were identified as β cells by their autofluorescence, as previously described (13). Groups of five to seven nondiabetic mice were analyzed at each time point. Mice showed geometric mean serum antibody titers to RRV of 1:110 and 1:730 at days 10 and 15 after RRV inoculation, respectively.

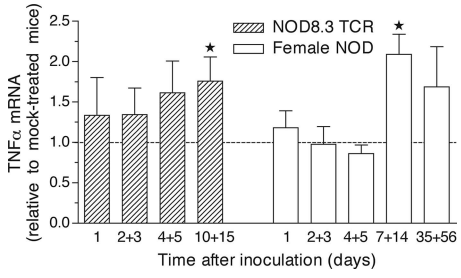

FIG. 8. Levels of islet TNF-α mRNA following RRV infection of insulitic mice. Groups of four to six mock- and RRV-inoculated nondiabetic mice were analyzed at each time point. The data from consecutive time points were merged for simplicity. The data for RRV-infected mice are presented relative to the mean mRNA level for mock-infected mice at the same time, which was standardized to 1.0 (dotted line). Significant differences between RRV- and mock-infected mice are marked with stars ($0.0033 \leq P \leq 0.036$). Mouse serum antibody titers to RRV were similar to those described in the legends to Fig. 6 and 7.

younger adult NOD mice infected with RRV under similar conditions (21). Apart from the test variable of mouse age (corresponding to the extent of insulitis), the only material difference between our earlier studies and those reported here is the RRV dose. Younger adult mice received a 10-fold-higher dose (2×10^7 FCFU) than older adult mice (1.8×10^6 FCFU) (21). However, we determined that inoculation of female NOD mice at 12 weeks of age with 10^7 FCFU of RRV did not materially affect the diabetes curves or seroconversion antibody titers obtained (data not shown). Preexisting severe insulitis or possibly other age- and insulitis-related events are important for the diabetes exacerbation caused by RRV.

RRV infection accelerates diabetes onset in all NOD and NOD8.3 TCR mice. The greater effect in females is consistent with their higher incidence of spontaneous diabetes (3, 54). Our diabetes survival curves indicate that RRV has a more pronounced effect on diabetes development in NOD8.3 TCR mice than NOD mice. Also, infection increases diabetes incidence in female NOD8.3 TCR mice, in female and male NOD8.3 TCR mice combined, and transiently in female NOD mice. Thus, RRV affects the timing of diabetes onset to a greater extent than incidence. The effect of RRV on diabetes manifests mainly in first 3 to 9 weeks after infection, suggesting that immune responses to infection are directly involved in diabetes modulation by RRV.

Extrapolation of findings from NOD mice to humans can be problematic. However, this model is highly relevant to human disease (3, 40, 46). The timing of rotavirus infection in relation to the prediabetic stage of at-risk children might similarly affect the outcome for their autoimmunity. This could help resolve the apparently conflicting findings regarding rotavirus infection and diabetes in Australian and Finnish children (6, 26). The majority (22 of 24; 92%) of the Australians were monitored to an older age (2.5 to 7 years) than the Finns (2 years) and so would have been more likely to encounter rotavirus at an advanced prediabetic stage during the study. In a further parallel, most children in the advanced prediabetic stage exhibited mild gastroenteritis or asymptomatic infection upon rotavirus reexposure. Similarly, mice with diabetes acceleration were asymptomatically infected with RRV.

T-cell molecular mimicry of rotavirus VP7 with HLA recognition regions in human GAD and IA-2 was proposed to explain the possible association between islet autoimmunity and rotavirus infection in children (26, 27). However, no corresponding mimicry has been demonstrated between murine determinants of MHC binding and rotavirus. Mimicry between rotavirus and these autoantigens in NOD mice is unlikely to occur since the critical human genes HLA-DR and HLA-DQ that confer diabetes susceptibility are absent (53). Any role for mimicry in these mice or humans was therefore not assessed in our experiments. However, molecular mimicry alone is considered to be unlikely to be sufficient to cause diabetes (10, 19). Consistent with this, our demonstration that RRV accelerates diabetes onset in NOD mice suggests that rotavirus mimicry with GAD and IA-2 might not be critical for rotavirus modulation of diabetes progression. Islet infiltration of diabetogenic $CD8^+$ T cells expressing the 8.3 TCR occurs early in the disease process in NOD8.3 TCR mice (54). Many $CD8^+$ T cells recruited to NOD mouse islets also recognize IGRP (2, 52). When activated with the cognate mimotope derived from IGRP (NRP), these cells respond to numerous NRP peptide analogues. No NRP mimics were identified in rotavirus proteins through protein database searches, so it is most unlikely that NRP molecular mimicry is involved in the diabetes exacerbation by RRV (1; B. S. Coulson, unpublished data).

The degree of diabetes acceleration and extent of intestinal RRV detection both followed the pattern NOD8.3 TCR > female NOD > male NOD. This implies a relation might exist between these parameters. Studies with a range of 50% virus shedding doses would help address this issue. Rotavirus infection in mice with invasive insulitis did not involve the pancreas, so direct pancreatic infection is not involved in RRV-induced diabetes acceleration. Possible mechanisms for β-cell damage induced by other potentially diabetogenic viruses (e.g., group B coxsackieviruses [CVB]), including cytolytic infection and destruction by antiviral immune responses, are thus unlikely to be relevant to RRV (19, 28, 50, 53). In contrast to RRV, pancreatic CVB infection is necessary for diabetes acceleration in insulitic female NOD mice (16, 43). In spite of this key difference in tropism, RRV and CVB show several parallel effects. Both viruses replicate in cultured islets but show a more restricted pancreatic involvement in vivo in virus-inoculated mice, and their role in type 1 diabetes relates to other factors apart from virus infection (11, 50; the present study). Notably, RRV and CVB show similar age-dependent effects on diabetes curves in female NOD mice (16, 43, 44, 51). Inoculation aged 4 weeks with diverse CVB strains protects against diabetes, whereas infection aged 12 weeks with virulent CVB at a low dose, or CVB of low virulence at a high dose, accelerates diabetes (50). It will be important to determine the effect of rotavirus dose on diabetogenicity.

Since RRV is not pancreatotropic in these older mice, alternative immunological mechanisms of diabetes exacerbation, such as those involving virus-induced proinflammatory mediator secretion, might be operating. These would be consistent with the observed relation between the level of serum antibody response to RRV and the degree of diabetes acceleration in insulitic mice. In addition to the degree of intestinal replication (see above), the strength of the murine immune response to rotavirus is a likely predictor of accelerated diabetes onset. This relation between rotavirus antibody titer and diabetes acceleration in NOD8.3 mice also suggests that $CD8^+$ T cells are involved. This is supported by the increased proportions of $CD8^+$ T cells in islets of male NOD mice after RRV infection. It is proposed that increased $CD8^+$ T-cell activity might produce the increased levels of islet TNF-α mRNA observed here after RRV infection. TNF-α induces MHC-I expression on β cells (9). The simultaneous increases in TNF-α mRNA and MHC-I at 10 days after RRV infection of NOD8.3 TCR mice imply that these could be functionally linked. It has been proposed that increased β-cell MHC-I creates a "fertile field" for the immune system by making β cells more recognizable (19). In the lymphocytic choriomeningitis virus model of virus-induced diabetes, MHC-I upregulation plays a central role in β-cell death (42). Thus, β-cell MHC-I upregulation might be important for RRV-induced diabetes acceleration. This could lead to increased immune surveillance, destruction of β cells and accelerated diabetes (9, 17, 19). TNF-α is elevated in

acute-phase serum from children with rotavirus gastroenteritis (30), and rapidly induced in the serum and intestine following human rotavirus infection in the pig model (5).

As for infant and young adult NOD mice, RRV infection in older NOD mice did not inhibit ongoing insulitis development (21). The trend for increased insulitis in older female and male NOD mice after RRV infection (Fig. 5) supports the accelerated diabetes observed in these mice and contrasts with the unaltered or slightly reduced insulitis in female NOD mice infected orally as infants or young adults, respectively (21). Overall, under conditions producing diabetes acceleration, RRV infection results in a trend for increased insulitis in NOD mice. Conversely, when RRV infection delays diabetes in NOD mice, little or no insulitis reduction is observed. The trend for increased insulitis in older female NOD mice after RRV infection was unrelated to the proportions of the $CD4^+$ and $CD8^+$ T lymphocytes present in the insulitis lesion. It will be important to determine the activation status of the major infiltrating cell types within the lesion. In male NOD mice, the trend for increased insulitis and islet $CD8^+$ T-cell proportions after RRV infection suggest that these cells might play a role in the diabetes acceleration observed. These sex-related differences in the effect of RRV on the composition of the insulitic lesion probably relate to the higher insulitis scores in RRV-infected NOD females than males at 16 to 17 weeks of age (Fig. 5). Any effect of RRV infection on components of the insulitic lesion might depend on the particular degree and nature of insulitis present at the time of infection.

Based on antigen detection, replication of EM mouse rotavirus was restricted to the intestine in infant BALB/c mice, suggesting that EM is less able to spread extraintestinally than other EDIM strains, including the original strain EW (7, 8). On days 3 to 5 after infection between 2 and 6% of the intestinal cells contained rotavirus antigen in our studies. These proportions are similar to those found previously (~1 to ~19%) in EW-infected infant BALB/c mice (39, 45). This suggests that EM replicates intestinally to an extent similar to that of EW. Although mouse numbers were small, EM exposure of older NOD mice was associated with diabetes of accelerated onset and increased incidence, an observation consistent with our findings in RRV-infected older mice. In the lymphocytic choriomeningitis virus mouse model, sequential infection with related arenaviruses is necessary to convert insulitis to overt diabetes (10). Older adult mice were infected once with RRV in our controlled experiments. However, serendipitous sequential infection of female NOD mice with RRV at 4 weeks of age and then EM as older adults also was associated with accelerated diabetes onset. This is notable since RRV infection at 4 weeks of age alone has no effect on diabetes incidence (21). Further studies of mouse rotavirus effects and the importance of sequential rotavirus infections are needed.

Our findings indicate that RRV rotavirus exacerbates murine diabetes by a mechanism that does not depend on direct pancreatic infection. Worldwide implementation of childhood vaccination with live attenuated rotaviruses is currently under way (4). The new animal models described here will be key tools in determining the effects of human rotaviruses and rotavirus vaccine strains on diabetes development.

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