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The Effect of Innate Immunity on Autoimmune Diabetes and the Expression of Toll-Like Receptors on Pancreatic Islets¹

Li Wen,^{2*} Jian Peng,* Zhenjun Li,[†] and F. Susan Wong[†]

Viral infections have previously been implicated as a trigger of autoimmune diabetes. In this study, we compared a viral mimic with other microbial components derived from bacteria in triggering diabetes development in C57BL/6-rat insulin promoter-B7.1 mice that do not normally develop diabetes. It is striking that only the viral mimic induced the development of diabetes in our model system. Further mechanistic studies suggest that diabetes is induced, in part, by the combination of direct recognition of this virus-like stimulus by pancreatic islets through the expression of the innate immune receptor, Toll-like receptor 3. In addition, the functions of APCs are up-regulated, and this could stimulate islet Ag-reactive T cells that will attack β cells leading to autoimmune diabetes. *The Journal of Immunology*, 2004, 172: 3173–3180.

Type 1 diabetes mellitus (T1DM)³ is an organ-specific autoimmune disorder that involves both genetic and environmental factors. Genetic factors clearly play a role in the development of diabetes, as the concordance rate of T1DM among monozygotic twins, studied in a number of populations, ranges from 23 to 53% (1–3). However, T1DM is increasing rapidly in many parts of the western world, especially in younger patients (4). Genetic risk factors may be necessary, but are not sufficient for the disease to occur, and environmental factors are thought to be important modifiers of susceptibility to type 1 diabetes (5, 6).

For many years, it has been suggested that viral infections play an important role in the precipitation of type 1 diabetes, both in animal models of the disease (7), as well as in humans (8). In animal models, they have been postulated to initiate disease and may do so by direct damage to the islets such as in the case of encephalomyocarditis virus (7), or by release of islet Ags (9) shown in mice, or by disruption of the immune balance as shown with Kilham rat virus in rats (7, 10). In addition, the islet β cells themselves produce a variety of antiviral responses for host protection, but in the process may also stimulate host immune-mediated and islet-directed damage (11).

dsRNA is produced in the process of viral replication of both DNA and RNA viruses (12). Poly(I:C) is a synthetic dsRNA that can stimulate immune responses similar to those produced by viral infections. Studies using poly(I:C) have shown a number of di-

verse effects that could modulate disease. Direct effects are seen in vitro within the islet that could stimulate changes leading to diabetes (11, 13). In addition, poly(I:C) has a very potent effect on APCs such as macrophages, and dendritic cells, in which poly(I:C) stimulates maturation and the production of IL-12 (14) and IL-15 (15). IFN- α is also markedly increased by poly(I:C) treatment (14, 16). The production of IFN- α in turn can stimulate a variety of cells mediating innate immune responses, including APCs such as dendritic cells and macrophages (17), as well as T cells (18) and NK cells (19).

In vivo, poly(I:C) can modulate the expression of autoimmune diabetes in animal models. This appears to be a dose-dependent phenomenon (20), as low dose poly(I:C) has been shown to protect against disease in the diabetes-prone Bio Breeding rat, whereas high doses precipitate the disease (21, 22). In the nonobese diabetic (NOD) mouse, in which few studies have been performed, the use of poly(I:C) has been shown to be protective (23). Poly(I:C) in the BDC2.5 transgenic mouse on a NOD background did not stimulate disease in a colony that develops a low incidence of diabetes (9).

Recently, it has been shown that the innate immune response is mediated through a number of receptors known as Toll-like receptors (TLRs) that are stimulated by different microbial stimuli (24). On binding the appropriate ligand, these receptors activate signaling pathways that lead to production of proinflammatory cytokines and up-regulation of costimulatory molecules. They also play a role in linking the innate and adaptive immune responses (25). The receptors are expressed on cells of the immune system, in particular APCs, but it is increasingly recognized that they are also expressed on other cells. In respect of antiviral responses, both viral and synthetic dsRNA interact with TLR3 (26).

Diabetes can be precipitated in rat insulin promoter (RIP)-B7.1 mice on a BALB/c genetic background by the use of poly(I:C) combined with insulin B9–23 peptide (27). The aim of this study was to investigate the role of TLRs and the means by which poly(I:C) combined with insulin can precipitate diabetes in mouse models that do not spontaneously develop diabetes.

Materials and Methods

Mice

C57BL/6 mice expressing B7.1 costimulatory molecules on pancreatic β cells under the RIP were generated, as described (28). These mice are designated B6/RIP-B7.1 mice. The RIP-B7.1 transgene expression was

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³ Abbreviations used in this paper: T1DM, type 1 diabetes mellitus; NOD, nonobese diabetic; NOR, nonobese resistant; PGN, peptidoglycan; RIP, rat insulin promoter; TLR, Toll-like receptor.

detected by PCR amplification of the B7.1 gene using genomic DNA isolated from tail biopsies. The mice were maintained in specific pathogen-free facilities at Yale University, and all the experiments were undertaken in accordance with approved Yale Animal Care and Use Committee protocols. BDC2.5 transgenic mice expressing the TCR of the BDC2.5 diabetogenic T cell clone were kindly provided previously by D. Mathis (Harvard University, Boston, MA). BALB/c and C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). NOD/Caj and nonobese resistant (NOR) mice were originally from The Jackson Laboratory and have been maintained for many generations at Yale University.

Reagents

Human (soluble) and bovine/porcine insulin (Lente) were purchased from Eli Lilly (Indianapolis, IN). The insulin B chain peptides aa 9–23 and 15–23 and the BDC2.5 mimotope peptide (29) were synthesized at the Keck facility of Yale University. Poly(I:C), poly(I), poly(C), LPS from Gram-negative bacteria (*Escherichia coli*), and peptidoglycan (PGN) from Gram-positive bacteria (*Staphylococcus aureus*) were purchased from Sigma-Aldrich (St. Louis, MO). Bacterial DNA sequence CpG oligonucleotide (tcc atg acg ttc ctg atg ct) was synthesized at the Keck facility of Yale University. CFA and IFA were also purchased from Sigma-Aldrich. Fluorochrome or biotinylated mAbs to CD80, CD86, CD40, I-A^b, and I-A^k (cross-reactive with I-A^{g7}) were purchased from BD PharMingen (San Diego, CA); anti-TLR3 was obtained from Biocarta; anti-rat secondary Ab was obtained from Caltag Laboratories (Burlingame, CA); and anti-I-A^b (212.A1) and anti I-A^{g7} (10.2.16) purified Abs were kindly provided by C. A. Janeway, Jr. (Yale University, New Haven, CT) IFN- α production was measured by an ELISA kit purchased from R&D Systems (Minneapolis, MN). The J774 macrophage cell line was kindly provided by K. K. Tong and N. Williams (University of Bristol). Apoptotic DNA ladder kit was purchased from Roche Applied Science (Indianapolis, IN).

Diabetes induction protocol

B6/RIP-B7.1 mice were used at 6–8 wk of age. They were divided into groups of 7–14 mice each. The mice were given one of a number of different microbial stimuli by injection (i.p.) daily for 7 days and then immunized (i.p.) with autoantigen (insulin or insulin B9–23 peptide) in the presence of IFA (protocol 1; Fig. 1) or CFA (protocol 2; Fig. 1) on day 14 postmicrobial stimulation. The concentrations of microbial stimuli used were 100 μ g/mouse (5 μ g/g body weight) for poly(I:C), 10 μ g/mouse (0.5 μ g/g body weight) for LPS, 10 μ g/mouse (0.5 μ g/g body weight) for PGN, and 10 nM/mouse for CpG. These concentrations were chosen based on the pilot experiments both in vitro (induction of activation markers on lymphocytes) and in vivo (without toxic effects such as weight loss due to anorexia). The concentrations of the Ag used were 25 μ g/mouse for insulin and 100 μ g/mouse for B9–23 peptide. Our pilot experiments using different concentrations of insulin (25, 50, and 100 μ g/mouse) had indicated that \geq 50 μ g insulin per mouse induced mild to severe hypoglycemia in the

recipients, and therefore 25 μ g was chosen. The mice were observed for up to 17 wk postimmunization for diabetes development by weekly screening for glycosuria, and the disease was confirmed by blood glucose ($>$ 250 mg/dl).

Cell lines

Diabetogenic cloned CD8 T cells (G9C8) that respond to the insulin peptide B15–23 (30) were maintained in Bruff's medium (Invitrogen, Carlsbad, CA) supplemented with 5% FCS and 5 U of IL-2 (supernatant of EL4 cell line) and cultured in 5% CO₂. They were fed every 2 wk with irradiated NOD islets isolated by collagenase digestion (30).

J774 macrophage cells were maintained in DMEM medium (Invitrogen) supplemented with 5% FCS, 2 mM glutamine (Invitrogen), and penicillin-streptomycin antibiotics. Before use, the adherent cells were removed from the culture flask using trypsin-EDTA and left to recover before use.

T cell proliferation and insulin Ab assays

TCR transgenic T cells (10⁴/well) were assayed for their islet responses, by culturing them for 72 h with irradiated (4000 rad) islet β cells obtained from handpicked pancreatic islets. Peptide responses were tested in BDC2.5 TCR transgenic or insulin peptide-reactive cloned CD8 T cells (10⁴/well) by culturing them with irradiated (3000 rad) NOD splenocytes and varying concentrations of the appropriate peptide for 72 h. [³H]Thymidine (PerkinElmer, Boston, MA) was added for the last 14 h, and the cells were harvested and counted in a beta plate counter (PerkinElmer). Serum samples from all the mice used in the study were collected before and after the experiments and assayed for insulin autoantibody, as described (27).

B cell purification

B lymphocytes were purified from splenocytes using the B cell isolation kit (Miltenyi Biotec, Bisley, U.K.), according to the manufacturer's instructions. Purity was $>$ 90% for all samples.

RT-PCR for the detection of expression of TLRs in pancreatic islets

Isolation of mouse pancreatic islets was as described (30). The islets were handpicked under a dissecting microscope, and this procedure was repeated two to three times until the islets were free of acinar tissue. The freshly handpicked islets were immediately lysed in TRIzol (Invitrogen), and RNA was prepared according to the manufacturer's instructions. cDNA was synthesized using oligo(dT) primer (Amersham Pharmacia Biotech, Piscataway, NJ) and Moloney murine leukemia virus SuperScript reverse transcriptase, according to the manufacturer's protocol. PCR was performed using specific primers for mouse TLR2, 3, 4, 5, and 9. Primer sequences were selected from the complete mRNA sequences in the National Center for Biotechnology Information database using MacVector software. PCR

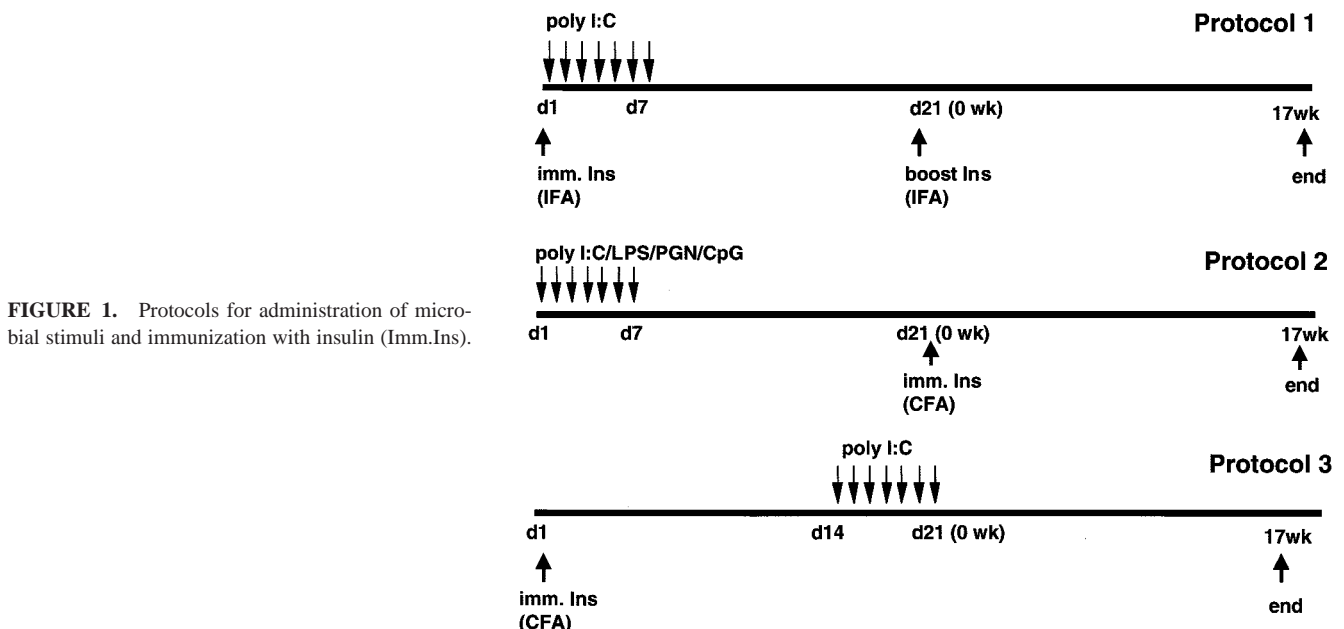


FIGURE 1. Protocols for administration of microbial stimuli and immunization with insulin (Imm.Ins).

conditions were standardized according to the parameters suggested by the MacVector program. Healthy human islets were received from the Islet Isolation Core Facility of Washington University (St. Louis, MO) School of Medicine headed by T. Mohanakumar and from the Juvenile Diabetes Research Foundation Human Islet Distribution Program at the University of Minnesota (Minneapolis, MN). The purified human islets were immediately washed with diethyl pyrocarbonate-treated PBS and lysed in TRIzol upon receipt. PCR was performed using specific primers for human TLR2, 3, 4, 5, and 9. The same approach was used for the selection of human TLR primers. PCR conditions were standardized according to the parameters suggested by the MacVector program. Primer sequences are available upon request.

mAb staining for TLR3 on islet cells

Following isolation of islets as described, the islet cells were dispersed using trypsin-EDTA (Invitrogen) for 5 min. They were cultured for 24 h in RPMI 1640 (Invitrogen) supplemented with 10% FCS, 2 mM glutamine, and penicillin-streptomycin antibiotics. The cells were divided into cultures using medium only or with 20 $\mu\text{g}/\text{ml}$ poly(I:C) added. After 24 h, the cells were washed and then stained sequentially with anti-TLR3 Ab, APC-conjugated anti-rat secondary Ab, PE-conjugated anti-I-A^b (for B6/RIP-B7.1 islets), or biotin-conjugated anti-I-A^k, which cross-reacts with I-A^{g7} (for NOD islets), followed by streptavidin-PE. This combination of Abs was chosen to avoid autofluorescence of islet cells in the FL-1 channel. This experiment was performed on two groups of islets; each was isolated from four mice, either B6/RIP-B7.1 or NOD mice.

Apoptotic DNA ladder assay

Handpicked pancreatic islets were cultured for 24 h (37°C, 5% CO₂) in the presence or absence of 100 $\mu\text{g}/\text{ml}$ poly(I:C) and 1 $\mu\text{g}/\text{ml}$ LPS. The islets were washed once with sterile PBS, and the DNA was isolated according to the instructions of the Apoptotic DNA Ladder Kit (Roche Applied Sci-

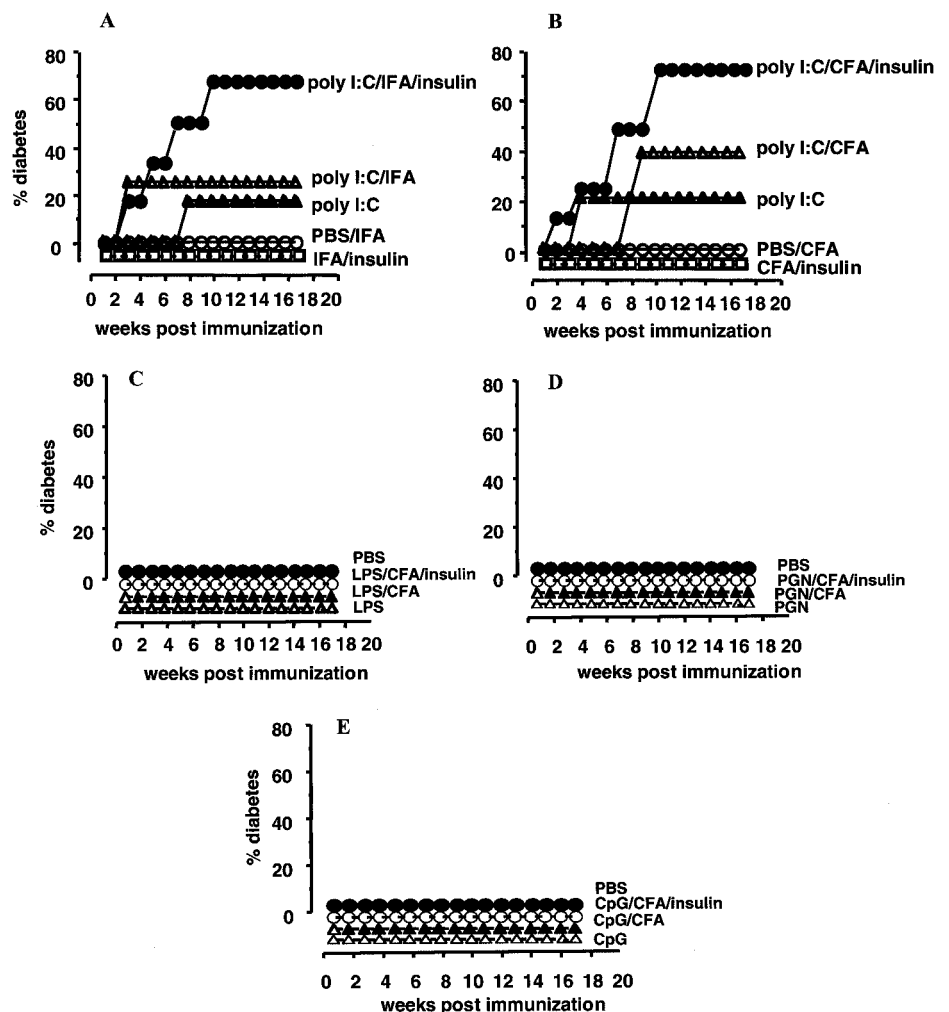
ence). The DNA ladder, which is a typical feature of apoptotic cells, was revealed by ethidium bromide in agarose gel.

Results

Poly(I:C), a viral mimic, can trigger the development of diabetes in B6/RIP-B7.1 mice

We have recently reported that poly(I:C), a mimic of viral dsRNA, can induce the production of insulin autoantibody and diabetes in conjunction with immunization of insulin B chain peptide 9–23 (B9–23) in mice with RIP-B7.1 expressed on an H-2^d genetic background (27). However, the insulin B9–23 peptide did not induce diabetes development in B6/RIP-B7.1 mice (H-2^b) using a similar experimental approach (data not shown). It is interesting that immunization with insulin protein, instead of B9–23, induced a high incidence of diabetes (>70%) in B6/RIP-B7.1 mice (Fig. 2A). The incidence of diabetes was not obviously affected by the type of adjuvant (CFA or IFA) used in the immunization (Fig. 2B). CFA together with poly(I:C) provoked ~40% of mice to become diabetic in the absence of insulin Ag (Fig. 2B). However, diabetes development was clearly associated with the administration of poly(I:C), as none of the mice developed diabetes without treatment with poly(I:C). In addition, CFA did not induce diabetes either alone (Fig. 2B), or in association with other stimuli (Fig. 2, C–E). Likewise, immunization with insulin (in either IFA or CFA) alone did not induce diabetes development in this experimental system (Fig. 2, A and B). However, ~20% of the mice developed diabetes with poly(I:C) treatment alone (Fig. 2, A and B). No sex

FIGURE 2. Diabetes development in B6/RIP-B7.1 mice postmicrobial stimulus treatment and insulin immunization. **A**, Poly(I:C) treatment and insulin immunization were performed as in protocol 1 (see Fig. 1). Five groups of mice were used in the experiment: poly(I:C)/IFA/insulin, $n = 11$; poly(I:C)/IFA, $n = 8$; poly(I:C), $n = 5$; PBS/IFA, $n = 5$; and IFA/insulin, $n = 5$. Diabetes was determined by monitoring of urinary glucose and confirmed by blood glucose >250 mg/dl (13.9 mmol/L). **B**, Poly(I:C) treatment and insulin immunization were performed as in protocol 2 (see Fig. 1). Five groups of mice were used in the experiment. Poly(I:C)/CFA/insulin, $n = 13$; poly(I:C)/CFA, $n = 10$; poly(I:C) alone, $n = 5$; PBS/CFA, $n = 5$; and CFA/insulin, $n = 5$. Diabetes was determined by monitoring of urinary glucose and confirmed by blood glucose >250 mg/dl (13.9 mmol/L). **C–E**, Diabetes development in B6/RIP-B7.1 mice post-LPS treatment (C), PGN (D), and CpG oligonucleotides (E), followed by insulin immunization (protocol 2). Four groups of mice were used in each set of experiments, and the number of mice per group varied between 5 and 8. The concentration of different stimuli was 100 $\mu\text{g}/\text{mouse}$ for poly(I:C), 10 $\mu\text{g}/\text{mouse}$ for LPS, 10 $\mu\text{g}/\text{mouse}$ for PGN, and 10 nM/mouse for CpG.



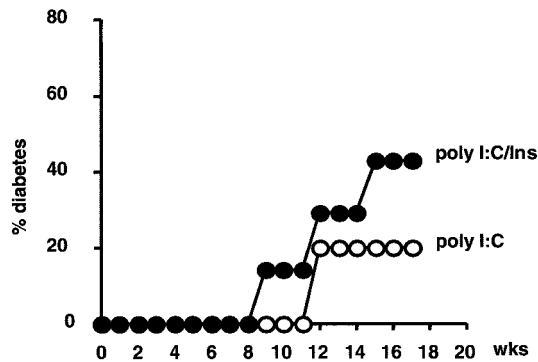


FIGURE 3. Diabetes development in B6/RIP-B7.1 mice postinsulin immunization and poly(I:C) treatment. Protocol 3 was used, and two groups of mice, with immunization ($n = 7$) and without immunization ($n = 5$), were used in the experiment.

preference was observed in the disease development in these mice, and B6/RIP-B7.1 mice rarely develop diabetes spontaneously (28).

Bacterial products failed to induce diabetes development in B6/RIP-B7.1 mice

To investigate whether bacterial products could also induce diabetes development in B6/RIP-B7.1 mice, we performed a series of experiments using other microbial stimuli before insulin immunization. These included bacterial products in the form of LPS from Gram-negative bacteria (*E. coli*) and PGN from Gram-positive bacteria (*S. aureus*), or bacterial DNA sequence (CpG). Surprisingly, no bacterial product used in the study was able to trigger diabetes development (Fig. 2, C–E).

ssRNA failed to induce diabetes development in B6/RIP-B7.1 mice

To study whether the ability of poly(I:C) to trigger diabetes development is dependent on the double-stranded structure, we performed groups of experiments using poly(I) or poly(C) together with insulin immunization. It is interesting that none of the mice treated with ssRNA developed diabetes (data not shown), despite the use of the same experimental conditions under which diabetes was induced by dsRNA. This suggests that diabetes induction by poly(I:C) is dependent on its double-stranded structure.

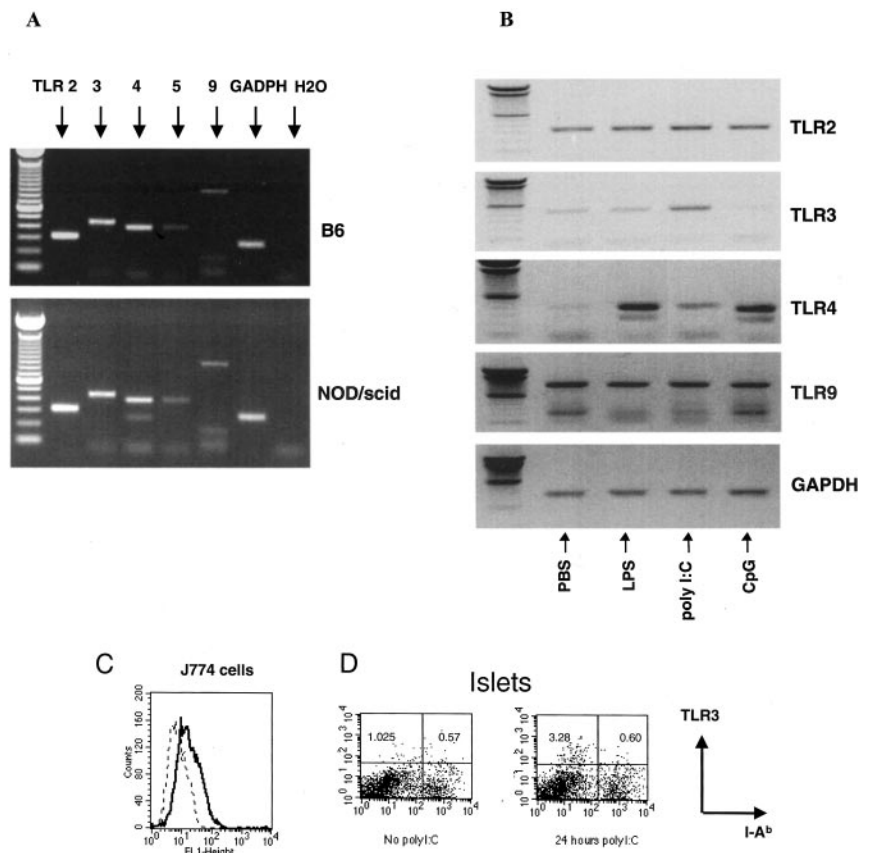
Poly(I:C) induces diabetes development regardless of the time of administration

In the experiments described above, we administered the poly(I:C) before insulin immunization. To study whether the time of poly(I:C) administration would affect disease development, we reversed the protocol (protocol 3; Fig. 1), i.e., insulin immunization was performed before the poly(I:C) injection in an additional set of experiments. The results demonstrate that the reversed protocol also promoted the disease development. However, the time of poly(I:C) treatment affects the incidence of diabetes development, as the reversed protocol induced a lower incidence of diabetes (Fig. 3).

Both mouse and human islets express various TLRs

TLRs are a family of receptors that recognize molecular patterns associated with microbial pathogens and induce innate and subsequently adaptive immune responses against microbial infection (24). TLRs are expressed mainly in APCs, and it is not clear whether tissue cells express TLRs and which TLRs are expressed in pancreatic islets under normal situations. It has been shown that poly(I:C) can induce changes within the islets (11, 31), but

FIGURE 4. TLR expression in mouse islets. *A*, Expression of different murine TLR mRNA was assessed using RT-PCR, as shown in ethidium bromide-stained gels. GAPDH (murine) was used as the internal control for normalization of RNA levels of tested samples, as indicated. RT-PCR was performed on oligo(dT)-synthesized cDNA, which was derived from total cellular RNA from freshly isolated pancreatic islets of the different mouse strains, as indicated. The m.w. marker is a 100-bp ladder. *B*, TLR expression assessed by RT-PCR in mouse islets following overnight treatment with microbial stimuli at the concentrations of 100 $\mu\text{g/ml}$ for poly(I:C), 1 $\mu\text{g/ml}$ for LPS, 10 $\mu\text{g/ml}$ for PGN, and 10 nM/ml for CpG. The m.w. marker is a 100-bp ladder. *C*, Anti-TLR3 staining of J774 macrophage cell line after 24-h incubation in culture with medium alone (broken line) compared with cells cultured in 20 $\mu\text{g/ml}$ poly(I:C) (solid line). *D*, Anti-TLR3 (APC) and anti-I-A^b (PE) staining of B6/RIP-B7.1 islet cells after 24-h incubation in culture with medium alone compared with cells cultured with 20 $\mu\text{g/ml}$ poly(I:C). This is representative of four experiments (two using B6/RIP-B7.1 islet cells, and two using NOD islet cells). The plots show an increase in the cells that stain for anti-TLR3 Ab that are not MHC class II positive (murine islet cells do not express MHC class II) in cells cultured with poly(I:C) compared with those cultured in medium alone.



whether poly(I:C) could interact with surface receptors had not been identified. To investigate this issue, we isolated pancreatic islets from various strains of mice, including BALB/c, C57BL/6, B6/RIP-B7.1, NOR, prediabetic NOD (young males), and NOD.SCID mice. All the islets were freshly handpicked two or three times to ensure purity. The same numbers of islets were used for RNA isolation, and the same quantity of RNA, measured by OD, was used for cDNA synthesis. RT-PCR was conducted using 2 μ l of 100 μ l of cDNA synthesized. It is interesting that TLR2, 3, 4, and 9 could be readily detected in the normal mouse islets, and TLR2, 3, and 4 showed a higher level of expression (Fig. 4A). The genetic background of mouse strains (BALB/c, C57BL/6, NOD, and NOR) does not seem to influence the expression pattern and level of TLRs in islets (data not shown); neither does the expression of the B7.1 transgene in the C57BL/6 strain (data not shown). Treatment with the various stimuli leads to up-regulation of the expression of the various TLRs, as shown (Fig. 4B). We also examined surface staining for TLR3 on the islets. The anti-TLR3 Ab demonstrated a low level of staining on the J774 macrophage line after 24-h stimulation with poly(I:C) (Fig. 4C). Following incubation of dispersed islet cells with 20 μ g/ml poly(I:C) for 24 h, a range of 1–3.3% of cells stained with anti-TLR3 (Fig. 4D). The cells stained with the anti-TLR Ab were not the resident APCs, as this population was distinguished from the islets by staining with anti-I-A^b Ab for islets from B6/RIPB7.1 mice or anti-I-A^k Ab for islets from NOD mice.

To investigate whether human islets also express detectable TLRs, we studied the pancreatic islets from healthy donors. It appeared that TLR3 was most strongly expressed in human islets (Fig. 5).

Poly(I:C) induces both B and T cell activation

To understand how poly(I:C) affects the immune system and triggers diabetes development in this model system, we treated splenocytes from B6/RIP-B7.1 mice with poly(I:C) and the other microbial stimuli and examined their activation markers by flow cytometry. Poly(I:C) considerably activated B cells by induction and/or up-regulation of their activation markers (Fig. 6A), as did the other microbial stimuli to a lesser extent. CD86(B7.2) was up-regulated more than CD80 (B7.1), and a maximal increase in level was seen after 24 h of stimulation (Fig. 6B). In addition, there

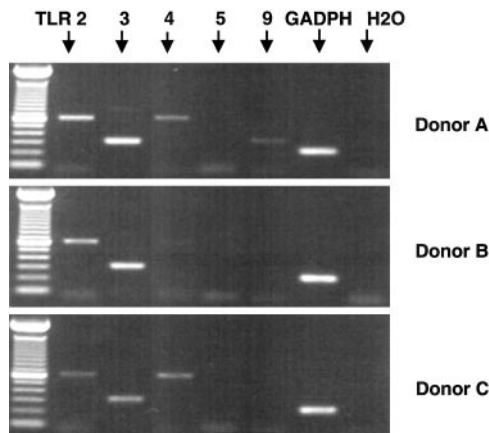


FIGURE 5. TLR expression in human islets. Expression of TLRs in human islets was assessed using RT-PCR, as shown in ethidium bromide-stained gels. GAPDH (human) was used as the internal control for normalization of RNA levels of tested samples, as indicated. RT-PCR was performed on oligo(dT)-synthesized cDNA, which was derived from total cellular RNA of purified healthy human pancreatic islets of different organ donors, as indicated, immediately upon receipt of overnight express delivery. The m.w. marker is a 100-bp ladder.

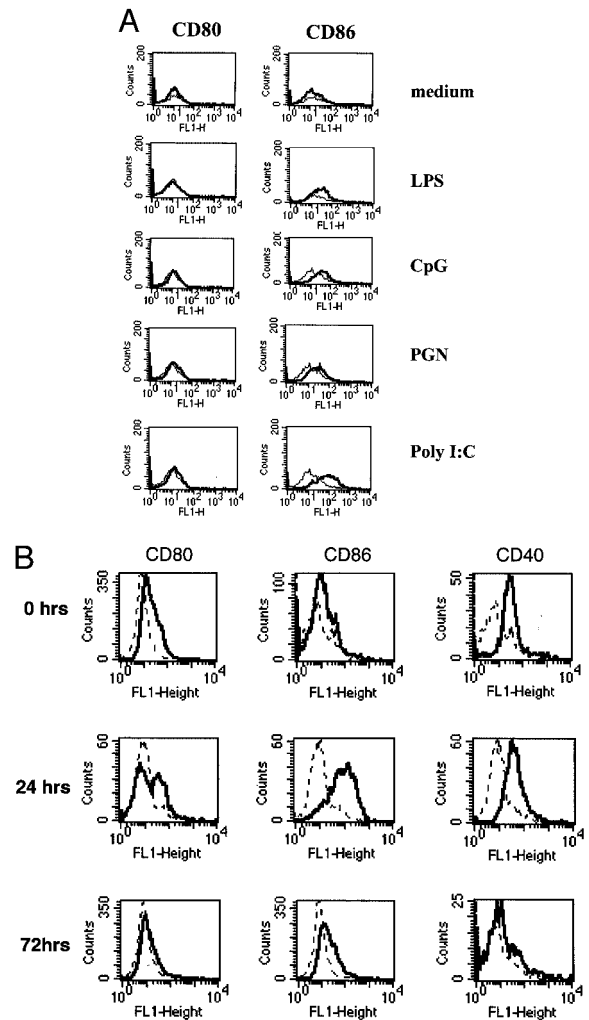


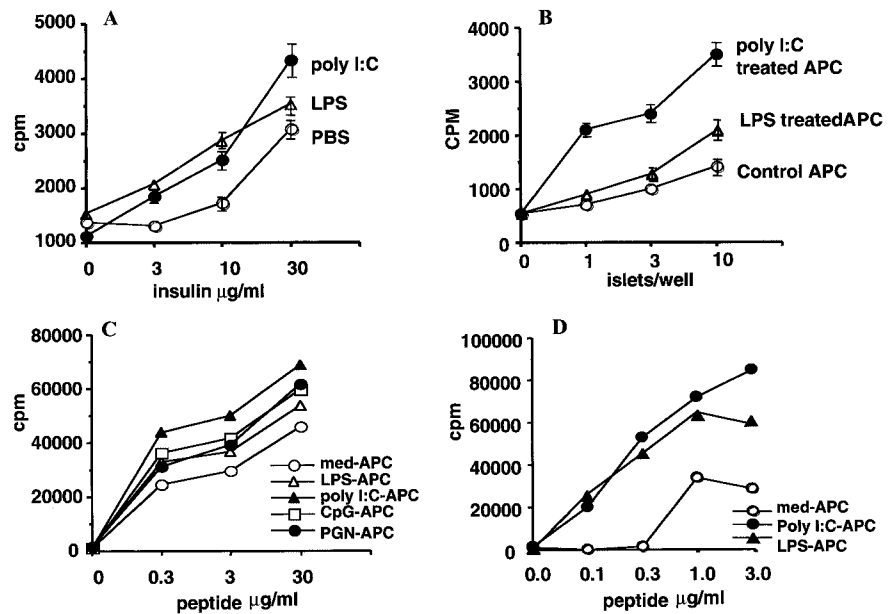
FIGURE 6. Expression of costimulatory molecules in B cells after microbial stimulation. *A*, Total splenic lymphocytes of different mouse strains were harvested and cultured overnight with various microbial stimuli, as indicated, at the concentrations of 100 μ g/ml for poly(I:C), 1 μ g/ml for LPS, 10 μ g/ml for PGN, and 100 μ M for CpG. The cells were extensively washed and stained with the B cell marker B220 (PE conjugated) in combination with either anti-CD80 (FITC conjugated) or anti-CD86 (FITC conjugated). Gated B220-positive cells were then analyzed, with the thick lines representing the costimulatory molecules and the thin lines showing the unstained controls. Up-regulation of CD86 was seen in all the strains tested (B6, B6/RIP-B7.1, and NOD), although the results from B6 mice are shown here. *B*, The time course of up-regulation of costimulatory molecules was further tested on purified B cells. Dotted lines represent controls with no Ab staining, and the solid lines indicate staining with the relevant Abs. This was representative of three experiments.

is also up-regulation seen on T cells following a similar stimulus (data not shown). As expected, poly(I:C) also promoted the maturation of splenic dendritic cells determined by the higher expression of MHC class I and II and CD86 costimulatory molecules (data not shown).

Insulin immunization increased the incidence of diabetes compared with diabetes induced by poly(I:C)/CFA or poly(I:C)/IFA alone. At the time of diagnosis of diabetes, spleen cells were tested for insulin reactivity, and it was consistently seen that poly(I:C) (and LPS) increased the T cell response to insulin (Fig. 7A), whereas diabetes development occurred only in poly(I:C)-treated mice.

To test the function of poly(I:C)-treated B cells as APCs, we performed [³H]thymidine incorporation proliferation assays using

FIGURE 7. Enhancement of Ag-specific T cell response. *A*, At the time that the experiment was terminated, splenocytes (from the mice of each group, which were treated with a microbial stimulus and insulin immunization) were isolated and tested for proliferation to insulin. At least five mice from each group were tested and shown as the mean \pm SEM of the results from mice treated with poly(I:C), LPS, or PBS. *B* and *C*, NOD splenocytes (as APC) were treated with the different microbial stimuli or medium alone (med-APC) for 24 h and used as APCs after irradiation and extensive wash. CD4 T cells from the BDC2.5 TCR transgenic mouse were used as responding T cells, and the Ag was irradiated male NOD islets (*B*) or BDC2.5 mimotope (*C*) as Ag presented on the different APCs. BDC2.5 T cell proliferation was measured by [3 H]thymidine uptake after 72-h incubation. *D*, Insulin-reactive cloned CD8 T cell proliferation to insulin B chain 15–23 peptide using irradiated B cells as APC that had been treated with 2 μ g/ml LPS, 20 μ g/ml poly(I:C), or cultured in medium alone. CD8 T cell proliferation was also measured by [3 H]thymidine uptake after 72-h incubation.



T cells with defined Ag specificity: BDC2.5 TCR transgenic CD4 T cells and G9C8 CD8 T cell clone. BDC2.5 T cells recognize a mimotope with high sequence homology to a GAD65 peptide (29), and the G9C8 CD8 T cell clone is specific for insulin B chain peptide 15–23 (32). However, both BDC2.5 and G9C8 T cells respond well to NOD islets. To test Ag processing and presentation function of poly(I:C)-treated APCs, we used poly(I:C)-treated and irradiated NOD splenic B cells (T-depleted splenocytes, in which B cells are the dominant population) as APCs and irradiated NOD islets as Ag. The same T-depleted splenic B cells without poly(I:C) treatment or treated with other microbial stimuli were used as control APCs. Poly(I:C) treatment markedly enhanced the ability of the APCs to process and present islet autoantigens to CD4 T cells (Fig. 7, *B* and *C*). Similar enhancement of proliferation to peptide was seen for CD8-cloned T cells (Fig. 7*D*). This may be due, in part, to the up-regulation of costimulatory molecules on the APCs by poly(I:C), as shown in Fig. 6.

IFN- α production induced by poly(I:C)

Splenocytes treated, *in vitro*, with poly(I:C) for 24 h, but not other microbial stimuli used in this study, produced \sim 50 pg/ml IFN- α . The level of IFN- α production does not appear to be influenced by the genetic background of mouse strains (data not shown). The

same treatment of islets induced only a marginal level of IFN- α (data not shown).

Poly(I:C) induced augmented apoptosis of pancreatic islets

Studies have shown that poly(I:C) could induce apoptosis of pancreatic islets (11, 13, 31). To examine whether this is also the case in our experimental system, we cultured the freshly handpicked pancreatic islets from B6/RIP-B7.1 mice in the presence or absence of 100 μ g/ml poly(I:C). We also cultured the islets with 1 μ g/ml LPS as non-dsRNA microbial stimulus control. Seventy islets were cultured under each condition. DNA from the cultured islets was purified, and ethidium bromide staining in agarose gel revealed a typical DNA ladder, the hallmark of apoptotic cells. As shown in Fig. 8, a faint DNA ladder could be detected from the islets that were cultured in medium only. This suggests that some apoptosis occurs in islets triggered either by the physical isolation procedure or by the same culture conditions. However, LPS-treated islets did not show enhanced apoptosis under the same culture condition (Fig. 8). In sharp contrast, poly(I:C) treatment induced increased apoptosis of pancreatic islets (Fig. 8).

Discussion

Immune responses during microbial infection are highly complex, and recent advances in understanding components of innate immunity have stimulated interest in the link with adaptive or acquired immune responses. Innate immune recognition of pathogens is usually mediated by a set of germline-encoded receptors that have evolved to recognize conserved molecular patterns shared by large groups of organisms. Recent studies have demonstrated that this recognition is mediated mainly through the TLR family (24, 33). TLR signaling can induce up-regulated expression of costimulatory molecules and production of proinflammatory cytokines and chemokines, thereby activating not only innate, but ultimately also adaptive immunity (34). Adaptive immune responses, mediated by T and B lymphocytes, recognize pathogenic Ags usually with high affinity through their rearranged receptors (TCR or B cell receptor) and protect the host after the initial phases of infection. However, these lymphocytes may also be involved in autoimmunity.

To examine the triggering of islet autoimmunity and diabetes development, we tested various microbial stimuli in conjunction

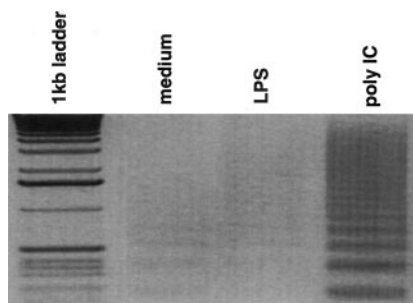


FIGURE 8. Augmentation of apoptosis of pancreatic islets by poly(I:C). Freshly isolated mouse pancreatic islets were cultured for 24 h in the presence or absence of 100 μ g/ml poly(I:C) or 1 μ g/ml LPS. The islets were washed once with sterile PBS, and the DNA was purified according to the instructions of the Apoptotic DNA Ladder Kit (Roche Applied Science). The DNA ladder was revealed by ethidium bromide staining in agarose gel.

with insulin autoantigen immunization in B6/RIPB7.1 mice that do not normally develop diabetes (28). We found that treatment with poly(I:C), a synthetic dsRNA, plus immunization with whole insulin protein induced a high incidence of diabetes in B6/RIP-B7.1 mice. Poly(I:C) treatment is required for disease development, as insulin immunization alone did not induce diabetes in these mice, whereas poly(I:C) treatment alone induced a low incidence of diabetes. It appears that poly(I:C) treatment before the immune response to insulin induced a higher incidence of diabetes (>70%) than using the reverse protocol (40%). The effect of this pretreatment with poly(I:C) primes the mice to increase the propensity to develop diabetes. This might be related to the fact that poly(I:C) triggers enhanced apoptosis of pancreatic islets, which releases islet autoantigens. These autoantigens can then be presented to the immune system. To test whether other microbial stimuli could have a similar effect to poly(I:C), we treated the mice with bacterial products and insulin immunization. It is striking that none of the mice developed diabetes when bacterial products (LPS, PGN, and CpG) were administered. Although there may be other factors involved, one difference is that the bacterial products (such as LPS) do not induce islet cells to undergo obvious apoptosis and, thus, there may not be the same availability of islet autoantigens to prime the immune system for the autoreactivity toward the pancreatic islets. The results of apoptotic DNA ladder assay appear to support this hypothesis. Our results indicate that infection with viruses might be a stronger environmental trigger for diabetes onset than bacterial infection.

TLR3 has recently been found to be the receptor for recognition of dsRNA (26). Although the expression of TLRs in general is thought to be mainly in cells involved in immune responses, TLR3 was shown to be expressed in lung and brain and up-regulated following LPS stimulation (26). As diabetes development in our model system is dependent on poly(I:C) treatment, and is not inducible, under these conditions, by other microbial stimuli, we examined the expression of TLRs in normal pancreatic islets. To our surprise, TLR2, 3, and 4 were strongly expressed in normal murine pancreatic islets. The expression of TLR5 and 6 was marginal (data not shown). The expression pattern was not affected by the genetic backgrounds of different mouse strains nor by the presence or absence of the B7.1 transgene. Stimulation of pancreatic islets with the TLR ligands up-regulated the expression of the corresponding TLRs. The up-regulation, per se, of these various TLRs, however, does not necessarily lead to development of diabetes.

We then investigated whether humans express a similar pattern of TLRs in pancreatic islets by analyzing human islets from three healthy organ donors. It is interesting that TLR3 showed the highest expression level in all the individuals studied. In mice, despite the high level expression of TLR2 and 4, *in vivo* treatment with their ligands (PGN and LPS, respectively) did not trigger diabetes development, whereas *in vivo* treatment with poly(I:C) (dsRNA, ligand of TLR3) provoked disease development. It has previously been shown that dsRNA can stimulate NF- κ B activation in islet cells, and this is not dependent on protein kinase R (13). This implies that an alternative mode of recognition is operative and could be related to stimulation of surface-expressed TLR3. Taken together, our data suggest that among various environmental factors, viral infection is a strong candidate for triggering diabetes development.

Insulin is an autoantigenic target in type 1 diabetes, as shown by the presence of autoantibodies in NOD mice (35) as well as the presence of pathogenic anti-insulin CD4 (36) and CD8 (32) T cells. Results shown in this work suggest that poly(I:C) treatment can up-regulate Ag-presenting capacity of APCs, in this case B

cells as well as other APCs, as shown by the increase in presentation of Ag to both islet-reactive CD4 and CD8 T cells. Mice on a B6 genetic background do not normally display any spontaneous anti-insulin T cell reactivity, nor do they develop anti-insulin autoantibodies. Although mice on a B6 background do not normally develop diabetes, they are able to respond to insulin after immunization (37, 38), indicating the presence of precursors, which under these circumstances could be stimulated to become pathogenic. However, even under the experimental conditions shown in this study, these mice do not produce insulin autoantibodies (data not shown).

Effects of poly(I:C) in relation to diabetes have been previously investigated mainly in the rat (20–22). It appears that the presence of predisposing MHC class II genes is a major factor in determining whether diabetes can be induced by poly(I:C) in normally non-susceptible rat strains (39). Paradoxically, the administration of poly(I:C) has suppressed diabetes when administered to NOD mice (23), and it was suggested that this was due to stimulation of suppressor T cells.

It is known that poly(I:C) can have direct effect within the islets by stimulation of dsRNA itself, such as induction of apoptosis via NF- κ B-dependent mechanisms and NO production (13, 31). Islets are also induced to produce chemokines such as IFN- γ -inducible protein-10 (11). In addition, stimulation through TLRs induces production of a number of cytokines. Viral infection and poly(I:C) as a viral mimic have both been shown particularly to induce IFN- α (17). IFN- α can accelerate and induce the onset of autoimmune diabetes when expressed as a transgene in islets in mice not genetically predisposed to diabetes (40). In the NOD mouse, IFN- α inhibits the development of diabetes (41). Interestingly, examination of histological sections showed that IFN- α could be detected in the pancreas of patients with type 1 diabetes (42, 43).

IFN- α is a potent stimulator of APCs, such as dendritic cells and macrophages (17, 44). However, in addition to the effects on APCs, IFN- α also stimulates CD8 memory T cells (18). In the context of this model of disease, both of these effects produced by poly(I:C) are likely to play a role in diabetes induction.

There are at least three effects of poly(I:C) that could stimulate the disease in a mouse model that does not normally develop spontaneous diabetes. First, poly(I:C) up-regulates APCs and augments their ability to present Ag to T cells. This augmentation occurs through up-regulation of the costimulatory molecules CD80 and especially CD86, as well as MHC class I and class II. Second, TLRs on the islets could respond directly to the poly(I:C) stimulus; the expression of TLR3 is seen both at the RNA level and on surface staining. Other investigators (11) and our own results presented in this study have shown that poly(I:C) can augment apoptosis of the islets, and it is possible that this may be mediated by TLR3. Finally, this all takes place in the presence of RIP-B7.1, which acts as an enhancer of the T cells that can damage the islet β cells, particularly by enhanced CD8 T cells. In this context, insulin can effectively prime T cells in the presence of poly(I:C) and provoke diabetes development. This implies that the quiescent endogenous insulin-reactive T cells can be augmented by poly(I:C), as a low incidence of diabetes is found in mice that have been treated with poly(I:C) alone. However, in the absence of poly(I:C), insulin immunization in this model system failed to induce the disease. In a different experimental system, insulin immunization alone has not been found to precipitate diabetes in the absence of B7.1 (45). It should be noted that islet β cells do not normally express B7 molecules. Although the presence of B7 molecules on the islet per se is not sufficient to induce diabetes, the added stimulus of poly(I:C) together with insulin can clearly cause diabetes. Our study does not suggest that autoimmune diabetes could be

easily induced in mice with a nonsusceptible genetic background. Rather, we would infer that even in a nongenetically susceptible mouse, autoimmune disease can be induced by a viral-like stimulus, increasing the availability of islet Ags and priming autoreactive T cells if an appropriate islet environment is present for the T cells to attack the β cells. In summary, we have shown that various TLRs are expressed within islets, but the ability to respond to the different microbial ligands does not have the same effect in provoking diabetes. It is clear that poly(I:C) as a viral stimulus has a different effect from the other microbial stimuli in diabetes development. Thus, infectious signals, per se, do not have equivalent effects in priming islet autoimmunity. We also demonstrate that among other effects, the activation of APCs by poly(I:C) could stimulate and increase the activity of islet-reactive T cells, which may play an important role in islet destruction. It is clear that more than one factor is required for the disease to occur, as certain signal transduction pathways are shared by other infectious stimuli, and yet these do not induce diabetes under the same conditions as was found for poly(I:C). Our data provide further mechanistic insights into how viral components may act to precipitate autoimmune diabetes.

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