

Acceleration of Type 1 Diabetes by a Coxsackievirus Infection Requires a Preexisting Critical Mass of Autoreactive T-Cells in Pancreatic Islets

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Coxsackievirus infections have been proposed as an environmental trigger for the development of T-cell-mediated autoimmune (type 1) diabetes by either providing a molecular mimic of the candidate pancreatic β -cell autoantigen GAD or inducing bystander inflammation in the pancreas. In this study in the NOD mouse model, we found that infection with a pancreatrophic coxsackievirus isolate can accelerate type 1 diabetes development through the induction of a bystander activation effect, but only after a critical threshold level of insulinitic β -cell-autoreactive T-cells has accumulated. Thus, coxsackievirus infections do not appear to initiate β -cell autoreactive immunity but can accelerate the process once it is underway. These findings indicate that the timing of a coxsackievirus infection, rather than its simple presence or absence, may have important etiological implications for the development of T-cell-mediated autoimmune type 1 diabetes in humans. *Diabetes* 49:708-711, 2000

T-cell-mediated autoimmune (type 1) diabetes in both humans and NOD mice is controlled by multiple susceptibility genes whose pathogenic functions can be modulated by various environmental factors (1-3). Coxsackievirus infections may represent one environmental factor that could contribute to type 1 diabetes development in genetically susceptible individuals (4). An antigenic epitope derived from the 65- and 67-kDa isoforms of GAD and proposed to be one of the earliest targets of diabetogenic CD4⁺ T-cell responses in NOD mice is characterized by a PEVKEK sequence also found within a peptide consisting of amino acids 28-50 from the coxsackievirus P2C protein (*Cox* sequence similarity peptide [ssp]) (5). Thus, one mechanism by which a coxsackievirus infection could contribute to type 1 diabetes is by providing a molecular mimic during replication that triggers a cross-reactive CD4⁺ T-cell response against the candidate β -cell autoantigen GAD. Arguing

against this molecular mimicry hypothesis was a report that GAD reactive T-cell responses were not enhanced in NOD mice after a coxsackievirus infection (6). Instead, this earlier study found that the coxsackievirus B4 Edwards strain (CVB4) can accelerate type 1 diabetes development in a T-cell receptor (TCR) transgenic stock of NOD mice in which virtually all T-cells are of the CD4⁺ BDC2.5 clonotype that recognizes a β -cell autoantigen other than GAD. This finding was interpreted to mean that rather than providing a molecular mimic of GAD, infection with the pancreatrophic CVB4 isolate contributes to type 1 diabetes development by stimulating a local inflammatory response that leads to subclinical levels of β -cell destruction and the subsequent release of normally sequestered antigens, which then trigger pathogenic autoreactive T-cell responses. However, this interpretation does not explain why, in that previous study, CVB4 infection failed to elicit type 1 diabetes development in standard nontransgenic NOD mice that are characterized by not only the presence of BDC2.5 clonotypic T-cells, but also a wide array of other β -cell autoreactive effectors, including some which may recognize GAD. This was a primary issue addressed by the present study.

RESEARCH DESIGN AND METHODS

Mice and virus infections. NOD/Lt and the previously described stocks (7,8) of T- and B-cell-deficient NOD-*scid* (official designation NOD-*Prkdc^{scid}*), and B-cell-deficient NOD-*Ig μ ^{null}* (official designation NOD-*Igh δ ^{mtC δ}*) mice are maintained by brother-sister mating at the Jackson Laboratory. Experimental mice were shipped to the University of Florida College of Medicine, where they were maintained in the Infectious Disease Control Facility for the virus infection studies. Mice were infected by intraperitoneal injection with 5×10^5 plaque-forming units of the previously described CVB4 Edwards strain (9), purified by a routine method (10). After CVB4 infection or injection with the saline vehicle, blood glucose values of >240 mg/dl on two occasions >24 h apart were considered to be diagnostic of type 1 diabetes development.

T-cell proliferation assays. Analyses of cellular immune responses to antigenic stimulation in vitro were performed with minor modification of previously described procedures (11). Briefly, splenocytes were plated at 5×10^5 cells per well in 96-well round-bottom plates in 200 μ l RPMI 1640 medium containing 10% fetal bovine serum. The indicated antigens were added at a concentration of 10 μ g/ml, and triplicate cultures were assayed. Cultures were pulsed with 1 μ Ci/well of [³H]thymidine over the final 18 h of a 5-day incubation period, then harvested and counted. Data are presented as the mean stimulation index, calculated by dividing the mean [³H]thymidine incorporation of splenocytes from an individual mouse cultured with antigen by the response in medium alone. The recombinant human GAD65 and the GAD p18 and *Cox* ssp used as antigens in these studies have been previously described (5,12).

Histology. Pancreases from mice assessed for insulinitis development were fixed in Bouin's solution and sectioned at 3 nonoverlapping levels. Granulated β -cells were stained with aldehyde fuchsin, and leukocytes with a hema-

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APC, antigen-presenting cells; CVB4, coxsackievirus B4 Edwards strain; ssp, sequence similarity peptide; TCR, T-cell receptor.

toxylin and eosin counterstain. A quantitative insulinitis score for some mice was then determined by the following criteria. Islets (at least 20 per mouse) were individually scored as 0, no lesions; 1, peri-insular leukocytic aggregates, usually periductal infiltrates; 2, <25% islet destruction; 3, >25% islet destruction; and 4, complete islet destruction. An insulinitis score for each mouse was obtained by dividing the total score for each pancreas by the number of islets examined.

RESULTS AND DISCUSSION

Although insulinitic destruction of pancreatic β -cells is limited in 6-week-old NOD females, they have already developed significant levels of GAD-reactive T-cells (13,14). B-cells represent a critical subpopulation of antigen-presenting cells (APC) for activating such GAD-reactive T-cells (15,16). A loss of APC capable of presenting major histocompatibility complex class II restricted β -cell antigens, possibly including GAD, to autoreactive CD4⁺ T-cells accounts for the diabetes and insulinitis resistance of the previously described stock of B-cell-deficient NOD.*Igμ*^{null} mice (7). However, it remained unknown whether subpopulations of APC other than B-cells had the capacity to generate mimicry epitopes of GAD from a coxsackievirus and hence trigger diabetogenic T-cell responses. Thus, 6-week-old standard NOD and B-cell-deficient NOD.*Igμ*^{null} females were infected with purified CVB4 to determine if it provided a cross-reactive antigen for amplifying GAD-reactive T-cell responses that could subsequently accelerate type 1 diabetes development and if so, the APC requirements for this process. To control for the possibility that the CVB4 strain could trigger type 1 diabetes by directly mediating lysis of pancreatic β -cells, T- and B-cell-deficient NOD.*scid* females were also infected. No consistent GAD-reactive T-cell responses were observed in either control or CVB4-infected NOD and NOD.*Igμ*^{null} females (data not shown). This finding was consistent with a previous report (6) that CVB4 infection of NOD mice does not provide an antigenic mimic capable of triggering cross-reactive T-cell responses to GAD.

Also similar to results previously reported by Horwitz et al. (6), standard NOD females infected with CVB4 or injected with the saline vehicle at 6 weeks of age did not differ in their extent of type 1 diabetes development by 12 weeks of age, the time at which we generally observe the first occurrence of spontaneous disease (Fig. 1). Similarly, CVB4 infection at 6 weeks of age did not trigger type 1 diabetes development in any NOD.*Igμ*^{null} (0 of 8) or NOD.*scid* (0 of 7) females over the same period of time. Histological examination at 3 weeks after CVB4 infection revealed massive pancreatitis and destruction of exocrine parenchyma in both NOD and NOD.*Igμ*^{null}, but not NOD.*scid*, females (Fig. 2). While various levels of insulinitis characterized endocrine islets of standard NOD females infected with CVB4, such lesions were absent in CVB4 infected NOD.*Igμ*^{null} females. These results indicate that exocrine rather than endocrine pancreatic tissue is damaged after CVB4 infection and that this process requires T- but not B-cells.

It has been proposed that islets in a pancreatic environment where exocrine inflammation has been induced by a CVB4 infection undergo subclinical levels of damage and release normally sequestered antigens that can subsequently trigger autoreactive diabetogenic T-cell responses (6). However, this hypothesis does not explain why CVB4 infection can induce type 1 diabetes in a TCR transgenic stock of NOD mice where virtually all T-cells are of the single BDC2.5 β -cell autoreactive clonotype (6), but not in standard 6-week-old

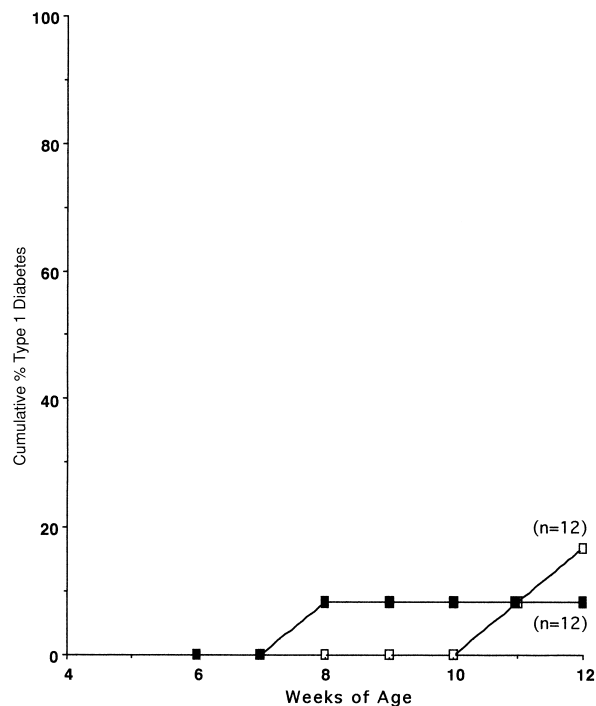


FIG. 1. Type 1 diabetes development is not accelerated in NOD mice infected with CVB4 at 6 weeks of age. NOD females were infected with CVB4 (■) or injected with saline vehicle (□) at 6 weeks of age and then monitored for type 1 diabetes development through 12 weeks, when spontaneous disease is normally first observed.

NOD females that harbor a wide array of diabetogenic T-cells. One possible factor contributing to this dichotomy is that the BDC2.5 TCR transgenic NOD stock develop insulinitis more rapidly than standard NOD mice (17). Thus, we hypothesized that a critical factor determining if CVB4 infection of the exocrine pancreas can accelerate type 1 diabetes development is whether a critical threshold of β -cell autoreactive T-cells had already accumulated in and around the islets. Provided sufficient numbers of insulinitic β -cell autoreactive T-cells were already present, their functional activities could then be directly amplified by inflammatory mediators produced in response to CVB4 infection of the exocrine pancreas.

If a CVB4 infection does not initiate, but rather enhances, ongoing β -cell autoreactive T-cell responses, then it should be possible to accelerate progression to overt type 1 diabetes in standard NOD mice by infecting them at a time when they have developed more extensive insulinitic lesions than those present at 6 weeks of age. As shown in Fig. 3, NOD females generally have higher levels of insulinitis at 8 than 6 weeks of age. Thus, to test the hypothesis outlined above, 8-week-old NOD females were either infected with CVB4 or injected with the saline vehicle. As a control, NOD.*Igμ*^{null} females were similarly treated at 8 weeks of age, since these mice have a greatly retarded rate of insulinitis development (7) but do not differ from standard NOD mice in the extent of CVB4-induced inflammation of the exocrine pancreas (Fig. 2). Over a 15-week period following CVB4 infection or saline treatment, no NOD.*Igμ*^{null} females developed type 1 diabetes (Fig. 4). Strikingly, after CVB4 infection at 8 weeks of age, 61.5% (8 of 13) of standard NOD females developed type 1 diabetes within the next 2 weeks (Fig. 4). In contrast, a signifi-

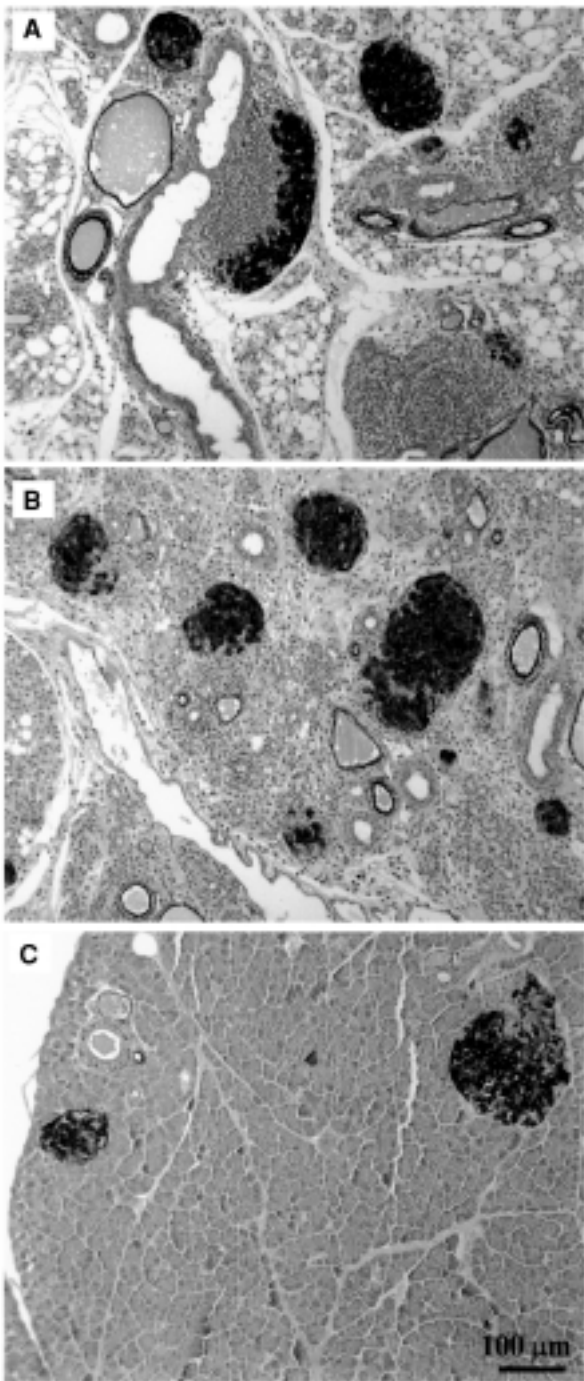


FIG. 2. Pancreatic histology of NOD, NOD.*Igμ^{null}*, and NOD-*scid* females infected with CVB4 at 6 weeks of age. Extensive destruction of pancreatic exocrine tissue was observed at 3 weeks after CVB4 infection in NOD (A) and NOD.*Igμ^{null}* (B) but not NOD-*scid* (C) females.

cantly longer period of time (8 weeks) was required before an equivalent level of spontaneously developing type 1 diabetes was observed in the saline-treated NOD females. Histological analyses of NOD and NOD.*Igμ^{null}* mice that remained free of type 1 diabetes for 10 weeks after CVB4 infection revealed equivalent high levels of pancreatic exocrine destruction. Collectively, these results indicate that

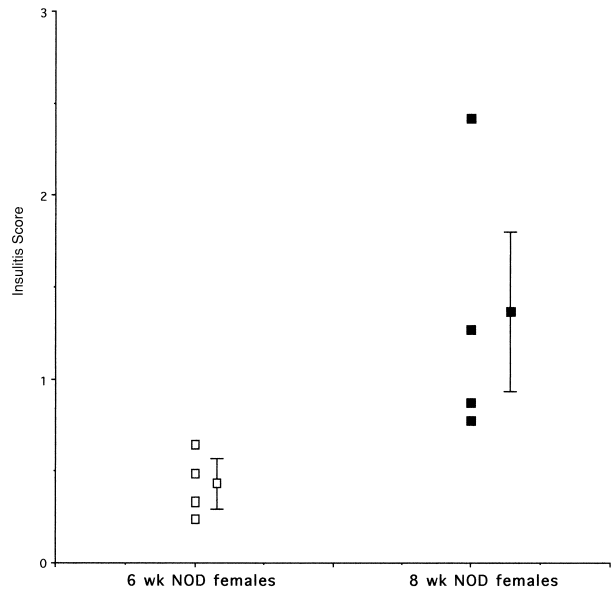


FIG. 3. NOD females are characterized by higher levels of insulinitis at 8 than 6 weeks of age. Insulinitis scores were determined as described in RESEARCH DESIGN AND METHODS for 4 randomly chosen 6- or 8-week-old NOD females. The data depict individual insulinitis scores as well as the mean insulinitis score \pm SE for each group. Mean insulinitis scores were significantly higher in 8- than 6-week-old NOD females ($P < 0.05$, Student's *t* test).

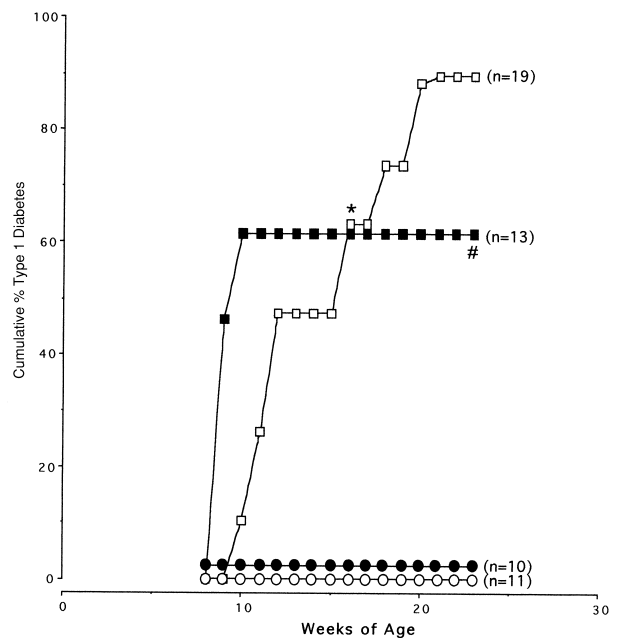


FIG. 4. CVB4 infection can greatly accelerate type 1 diabetes development in a subset of NOD females with more advanced levels of insulinitis than that present at 6 weeks of age. NOD (\square , \blacksquare) and NOD.*Igμ^{null}* females (\circ , \bullet) were infected with CVB4 (\bullet , \blacksquare) or injected with the saline vehicle (\circ , \square) at 8 weeks of age and then monitored over a 15-week follow-up period for type 1 diabetes development. *Significantly faster rate of type 1 diabetes development through 15 weeks of age in NOD females infected with CVB4 than injected with saline at 8 weeks of age ($P = 0.0002$, Kaplan Meier life-table analysis). #Significantly retarded rate of type 1 diabetes development from 15 to 23 weeks of age in NOD females infected with CVB4 than injected with saline at 8 weeks of age ($P = 0.0083$, Kaplan Meier life-table analysis).

the inflammatory mediators produced in response to a CVB4 infection of the exocrine pancreas can provide a bystander activation effect that accelerates the development of type 1 diabetes, but this can only occur after the accumulation of a critical threshold level of insulinitic β -cell-autoreactive T-cells.

It is interesting to note that the 5 NOD females that did not develop type 1 diabetes within the 2 weeks after CVB4 infection (presumably due to lower levels of pre-existing insulinitis than those that did) remained disease-free through 23 weeks of age. Indeed, while the CVB4-infected NOD females were characterized by an accelerated rate of type 1 diabetes development through 15 weeks of age, their disease rate after that time was significantly less than that of saline-treated controls (Fig. 4). This finding suggests the interesting, but at this point still unproven, possibility that, similar to a number of other viruses (3), CVB4 infection before the development of a critical threshold of insulinitis might actually block the development of type 1 diabetes in NOD mice through a nonspecific immunostimulatory mechanism.

In conclusion, coxsackievirus infections appear not to initiate β -cell autoreactive immunity but can accelerate the process once it is underway. These findings in a mouse model system indicate that the timing of a coxsackievirus infection in humans may be the most important factor in determining whether it can catalyze the development of T-cell-mediated autoimmune type 1 diabetes.

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