Islet T Cells Secreting IFN-γ in NOD Mouse Diabetes: Arrest by p277 Peptide Treatment

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Introduction

Type I, insulin-dependent diabetes mellitus in NOD mice, as in humans, develops as the result of β-cell destruction directed by autoimmune T cells [1]. A range of target antigens has been described in NOD IDDM [2] and it is hoped that some of them might be used to develop immunologically specific treatments for the disease.

We have reported that a single injection of p277, a peptide derived from the sequence of the human hsp60 molecule, into NOD mice, even late in the course of insulitis, can arrest the progression of the autoimmune process [3, 4]. Administration of peptide p277 could also prevent the development of autoimmune diabetes induced by an ultra-low dose of the β-cell toxin streptozotocin [5]. Effective p277 peptide therapy could be attributed to a switch from ‘pro-inflammatory’ Th1-type autoimmunity to ‘anti-inflammatory’ Th2-type autoimmunity, detected specifically [6]. However, it has been difficult to enumerate the cytokine-producing T cells in the islets in the natural course of the disease and there has been as yet little documentation of the effects of peptide p277 or other immunological treatments on the islet-associated T cells that actually cause the disease. This paper reports the results of our adaptation of the ELISPOT assay [7], making it possible to count the islet-infiltrating T cells that produce IFN-γ, TNF-α or IL-4. In this study, we isolated islet-infiltrating T cells and activated them to secrete their cytokines in vitro by using a mitogenic anti-CD3 antibody. This T-cell specific stimulation allowed us to sample the cytokine secretion of islet CD4+ and CD8+ T cells irrespective of their antigen specificities.

Here we report that intraislet IFN-γ producers predominate during the development of IDDM. Moreover, we found that arrest of the disease by p277 peptide therapy is associated with abrogation of the IFN-γ response in the islets.

Materials and Methods

Mice

NOD/Lt mice were raised at the animal facilities of the Weizmann Institute of Science from breeders kindly supplied by Dr. E. Leiter from Jackson Laboratories (Bar Harbor, ME). The mice were housed under specific pathogen-free conditions. The incidence of disease in our colony was about 80% in...
females and 40% in males by 28 weeks of age. Mice were bled from the tip of the tail and blood glucose levels were measured using the ‘Companion 2’ blood-glucose sensor (MediSense Inc., Waltham, MA). Animals were considered diabetic if their blood glucose was >11 mmol/l on two consecutive weekly determinations. Non-diabetic mice of both sexes from 1 to 4 months of age were used for islet isolation in these studies.

**T-cell clones**

Two CD4+ NOD T-cell clones were used to calibrate and validate the ELISPOT assay. C1 was a Th1 clone specific for the p277 peptide of hsp60 that secreted IFN-γ but not IL-4 in response to p277 stimulation, measured by a standard ELISA [8]. Clone C2, in contrast, produced IL-4 but not IFN-γ when stimulated by hsp60. C1, but not C2, could adoptively transfer hyperglycemia and insulinitis to syngeneic mice (D. E., in preparation).

**Antibodies**

Anti-mouse cytokine antibody pairs were rat monoclonal BVD4-1D11 (capture) and BVD6-24G2 (detection) for IL-4; and R4-6A2 (capture) and XMG1.2 (detection) for IFN-γ (PharMingen, San Diego, CA). For TNF-α, goat anti-mouse TNF-α (R&D Systems, Minneapolis, MN) was used as the capture and rabbit anti-mouse TNF-α (PharMingen) as the detecting antibody. The antibodies used for detection were purchased biotinylated. For T-cell activation, we used monoclonal hamster anti-mouse CD3ε antibody 145-2C11 (ATCC, Rockville, MD) purified from hybridoma tissue culture supernatant by caprylic acid, ammonium sulfate precipitation and dialysis [9].

**Preparation of islet cells**

Pancreatic islets were isolated by the collagenase method [10] with some modifications. Mice were killed and 2 ml sterile cold collagenase P (Boehringer Mannheim, Mannheim, Germany), 0.5 U/ml in Hank’s balanced salt solution (HBSS), were injected into the common bile duct. Pancreata from five to 10 mice were collected aseptically on ice, and incubated in 37°C for 40 min to digest the connective tissue. The tissue was then dispersed by shaking and separated by centrifugation (800 × g, 10 min) on a three-layer discontinuous gradient of Histopaque (1119–1085–1065; Sigma Diagnostics, St. Louis, MO). The islets were collected from the lower interface and counted to estimate the number of islets per mouse. To obtain islet cells in suspension, the islets were digested by 0.25% trypsin–0.05% EDTA in Puck’s saline (Biological Industries, Beit Haemek, Israel) for 10 min at 37°C and the cells were dispersed by gentle pipetting into a single-cell suspension. The cells were washed three times in RPMI-1640 medium, supplemented with 10% fetal calf serum (FCS; Biological Industries, Beit Haemek, Israel), 2 mM l-glutamine, 10 mM HEPES and antibiotics (complete medium), and counted. To determine the numbers of leukocytes in the cell suspension, samples of 0.2 ml, containing 8×10⁵ cells, were centrifuged on slides (2,500 rpm, 5 min) using a Shandon Elliott cyt centrifuge (Shandon Scientific Co., London, UK). The slides were stained with Gill’s hematoxylin and the leukocytes could easily be distinguished by their size and morphology from the large islet epithelial cells. At least 400 cells were counted, and the numbers of mononuclear leukocytes were calculated as a percentage of the total cells. To confirm that the cells had originated from islets, we detected β-cells by immunoperoxidase staining for insulin using guinea-pig anti-insulin antibody, peroxidase-labelled rabbit anti-guinea-pig antibody (Zymed Laboratories Inc., San Francisco, CA) and 3,3′-diaminobenzidine (DAB) as substrate (Sigma).

**Cytokine ELISPOT assay**

The gel substrate method was used [7]. Sterile ELISA 96-well flat-bottomed plates (Corning Inc., Corning, NY) were pre-coated with capture anti-cytokine antibodies (2 μg/ml in PBS) by incubation overnight at 4°C. The plates were washed with PBS and blocked with 100 μl complete medium for 1 h before adding the islet cells. Islet cells were plated at densities of 4×10⁶, 2×10⁶, 10⁵ and 5×10⁴ cells per well in duplicate. Spleen cells were seeded at densities 2.5×10⁵, 10⁵, 5×10⁴ and 10⁴ cells per well. Cloned T cells were placed at 10-fold dilutions beginning at 10⁵ per well. The plates were centrifuged (50×g, 4 min) and incubated for 18 h at 37°C, in 5% CO₂ in air. The cells were removed from the wells by washing with PBS plus 2% FCS, the wells were blocked with 10% FCS in PBS (1 h, 37°C) and the test detection antibodies (4 μg/ml in PBS plus 2% FCS) were added for 2 h. Streptavidin-conjugated alkaline phosphatase (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), 1:500 in PBS plus 2% FCS was added for 40 min at 37°C. The wells were washed and each well received 100 μl of the pre-warmed substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 2-amino-2-methyl-1-propanol (AMP) buffer with 0.6% agarose (Sigma). Positive spots were blue spots up to 0.1 mm in diameter, identified by their round shape and diffuse borders, that were counted the next day using an inverted microscope (Olympus CK2, Olympus Optical Co., Tokyo, Japan) with a 10× lens. Artefactual precipitates, much smaller in size and of irregular shape, were ignored. The results are expressed as spot-forming cells (SFC) per islet or per total leukocytes for each cytokine. Wells with mismatched detection antibodies, without capture antibodies or without cells were used as negative controls, and background spots (usually no more than five) were subtracted.
Activation of T cells

In the absence of activation, there was almost no spontaneous secretion of IL-4 and IFN-γ, and only few TNF-α SFC could be seen. T cells were activated using the anti-CD3 antibody 145-2C11 absorbed to the test plate (5 μg/ml) together with the test cytokine capture antibody. This anti-CD3 antibody recognizes the ε-chain of the mouse T cell receptor complex and can activate T cells to secrete cytokines in the absence of accessory cells [11, 12]. The absorbed anti-CD3 antibody works by cross-linking the CD3 complex without causing the T cells to cluster together and, unlike mitogenic lectins, is suitable for use in the single-cell ELISPOT assay. In preliminary studies, consistently higher numbers of IL-4 and IFN-γ SFC were detected using the anti-CD3 antibody compared to the lectin concanavalin A.

Acceleration of IDDM by cyclophosphamide

Groups of 20 NOD males were injected i.p. either with 200 mg/kg cyclophosphamide (ISOPAC, Sigma) in PBS or PBS alone, twice with a 10 day interval [13]. Three days after the second injection, five normoglycemic mice of each group were sacrificed for islet isolation, and the remaining mice were monitored weekly for blood glucose to confirm the acceleration of disease.

Peptide therapy

Peptide p277, residues 437–460 of the human hsp60 molecule with valines in place of cysteines [4, 5] at positions 6 and 11 (VLGGVALLRVIPALDSLTPANED), and control peptide MT-p278 [6], residues 430–446 of M. tuberculosis hsp65 (EGDEATGANIVKALEA), were prepared by standard Fmoc synthesis, using an automated multiple peptide synthesizer (AMS 422, Abimed, Langenfeld, Germany) as described [3]. The peptides were purified by reverse-phase HPLC and their sequences were confirmed by amino-acid analysis. The peptides were diluted in phosphate-buffered saline (PBS) and emulsified in incomplete Freund’s adjuvant (IFA; Difco Laboratories, Detroit, MI). Groups of 30 female NOD mice, 3 months of age, received 100 μg of peptide or PBS in IFA s.c. as a single dose. Forty-five days after treatment, the islets and spleen cells were obtained, the leukocytes counted and the cytokine ELISPOT assay was performed.

Statistical analysis

Numbers of SFC were compared using the two-tailed unpaired Student’s t-test. The cumulative incidence of diabetes was compared by Fisher’s exact test and its association with the numbers of cytokine producers was analysed by Pearson’s correlation.

Results

Insulitis and incidence of IDDM in male and female NOD mice

Figure 1 shows the cumulative incidence of diabetes (A) and quantifies the degree of insulitis by the numbers of leukocytes isolated per islet (B) monthly in male (—○—) and female (—●—) NOD mice at monthly intervals.
It is known that the progression of peri-islet insulitis to penetrating intra-islet insulitis precedes the outbreak of clinical diabetes [15], so a qualitative change in the infiltrating cells seems to be critical for β-cell destruction. Attention has been called to the importance of the Th1–Th2 dichotomy in the disease process [16]. We therefore adapted the ELISPOT cytokine assay of T-cell behaviour to probe the islet-associated leukocyte populations and investigate representative Th1 and Th2 cytokines during the autoimmune prodrome.

Calibration of the ELISPOT assay

Before applying the ELISPOT assay to islet T-cell populations, it was necessary to know which cytokines could be assayed and whether there was a sensitivity bias for one or another cytokine intrinsic to the assay. Furthermore, we wished to learn whether T-cell activation using the anti-CD3 antibody might change the cytokine phenotype. We used known T cell clones to validate the system. Figure 2 shows the results of activating prototypic Th1 and Th2 clones with anti-CD3 and assaying the percentage of the cultured T cells that could produce IFN-γ, IL-4 or TNF-α spots in the ELISPOT assay. It can be seen that about 30% of the Clone 1 cells could produce a detectable IFN-γ spot and no IL-4 spots, and about 25% of the Clone 2 cells could produce a detectable IL-4 spot and no IFN-γ spots. Both clones produced TNF-α spots (approx. 15–50%). Thus we could conclude that the ELISPOT assay appeared to be about equally sensitive for detecting IFN-γ and IL-4 producers. Moreover, the cytokine phenotypes of the clones revealed by anti-CD3 activation were identical to those obtained by stimulation with antigens presented by antigen-presenting cells and measured by ELISA (not shown). With regard to other cytokines, the ELISPOT assay was found not to be sensitive for enumerating IL-2 or IL-10 producers (not shown), and the reagents needed to develop an ELISPOT assay for TGF-β were not available. Therefore, our results are limited to IFN-γ, IL-4 and TNF-α.

Islet IFN-γ SFC accumulate with age

To document the natural development of insulitis, cytokine-producing islet T cells were assayed in normoglycemic male and female mice at monthly intervals during the first 4 months of age. No spontaneous cytokine-secreting cells could be detected in the islets, except for a few TNF-α SFC. However, numerous cytokine producers were revealed upon activation with anti-CD3.

Figure 3 shows that the numbers of potential IFN-γ SFC within the islets increased with age in both...
females and males. A strong correlation between IFN-γ SFC per islet and the cumulative incidence of IDDM (see Figure 1) was found (r=0.819, P=0.0003), and this age-dependent accumulation of IFN-γ producers was more rapid in females. The frequencies of IFN-γ SFC calculated per 10^4 islet leukocytes were also much lower in male mice (data not shown). Thus, the relative resistance of males to the development of IDDM was associated with a slower accumulation of IFN-γ-secreting T cells. TNF-α secretors in the islets did not appear earlier in the female than in the male mice. The numbers of IL-4 SFC were small in the islets of both the males and the females.

**Cyclophosphamide induces intraislet IFN-γ producers**

If the development of diabetes is functionally related to T cells that secrete IFN-γ, then accelerated induction of IDDM in males should be associated with elevated numbers of islet IFN-γ SFC. This was tested by treatment with cyclophosphamide. Administration of cyclophosphamide to 2-month-old NOD males in two doses of 200 mg/kg i.p. resulted in accelerated diabetes (Figure 4A). A control group injected with PBS remained normoglycemic, while 60% of the cyclophosphamide-treated mice became hyperglycemic within 2 weeks of the second injection. Three days after the second injection, an eight-fold increase in IFN-γ SCF was observed in the cyclophosphamide-treated (yet normoglycemic) mice compared to the controls (Figure 4B). TNF-α producers were present in the islets before the administration of cyclophosphamide and the increase of TNF-α SFC after treatment was not impressive. Similarly, there were no marked changes in IL-4 producers. Thus the acceleration of IDDM was associated mainly with upregulation of IFN-γ-secreting T cells in the islets.

**Effect of p277 treatment on T-cell cytokines in the islets**

Treatment of NOD female mice with the p277 peptide administered in IFA subcutaneously was found to arrest the progression of β-cell destruction [3–6], associated with a switch in splenic anti-p277 T-cell responses from IFN-γ to IL-4 secretion measured in vitro by ELISA [6]. However, to affect the disease process, p277 peptide treatment would have to affect the T cells infiltrating the islets.

To test whether p277 administration can influence insulitis globally, we treated 12-week-old NOD female mice with peptide p277 or MT-p278 and observed the effects of treatment on the incidence of diabetes at 7 months of age. Figure 5 shows that treatment with peptide p277, as reported previously, led to a significant decrease in the incidence of mice developing diabetes (P<0.03). Treatment with MT-p278, in contrast, did not affect the development of the disease. Figure 6A shows the islet-associated T cells producing IFN-γ or IL-4 in response to anti-CD3 activation 45 days after peptide treatment. It can be seen that treatment with peptide MT-p278 did not lead to any significant reduction in the IFN-γ secretors, just as this treatment failed to affect the progression of IDDM (Figure 5). Administration of peptide p277, however, induced a marked decrease in the numbers of IFN-γ-producing islet T cells. In contrast, there was no significant effect of p277 peptide treatment on TNF-α producers (not shown). The global decrease in IFN-γ-producers, which was observed in the islets, did not occur in the spleen. Figure 6B shows that the numbers of splenic T cells activated by anti-CD3 to secrete IFN-γ or IL-4 was not affected by treatment with p277 or MT-p278. Thus, IFN-γ SFC disappeared from the pancreas selectively.
Figure 7 shows that p277 peptide treatment led to an increase in the number of islets per mouse and to a decrease in the numbers of leukocytes per islet detected 2 and 6 weeks after treatment. Thus, the reduction in the IFN-γ producers in the islets was accompanied by reduction in the magnitude of the insulitis and by a relative increase in islet numbers.

Discussion

The aim of this study was to enumerate islet T cells secreting cytokines thought to be important in the development of autoimmune type 1 diabetes in NOD mice, i.e. IFN-γ, TNF-α and IL-4.

IFN-γ has been reported to inhibit the secretion of insulin and to be cytotoxic to islet cells in vitro, particularly in combination with TNF-α/β and IL-1-α/β [17–19]. IFN-γ can upregulate the expression of ICAM-1 [20] and MHC class I [21] and MHC class II molecules [22]. IFN-γ also exerts a strong positive feedback on Th1-type T-cell effector functions [23]. IFN-γ, in short, can make the islets better targets and the T cells more responsive. Indeed, BALB/c mice, mice otherwise not known to be prone to IDDM, develop autoimmune diabetes when they express transgenic IFN-γ in their islets under the direction of an insulin promoter (RIP-IFN-γ) [24]. Anti-IFN-γ antibody can prevent this transgenic diabetes [25], just as it can downregulate the diabetes developing spontaneously in NOD mice [26], or that induced by low-dose streptozotocin in C57BL/KsJ mice [27]. Despite these findings, however, it was recently shown that IDDM could still develop in NOD mice in which IFN-γ was genetically deleted [28]. The mechanism responsible for IDDM in the absence of IFN-γ was not clarified in that study, but one can imagine that the redundancy of pro-inflammatory cytokines might allow the immune system of these mice to organize alternative pathways for destructive autoimmunity. Be that as it may, IFN-γ does seem to be a critical factor for the IDDM process, provided the molecule is available to the immune system.

The pathogenic role of TNF-α in autoimmune diabetes is less clear than that of IFN-γ. TNF-α has been shown in vitro to inhibit the release of insulin from β-cells [17] and in vivo to augment the cytotoxicity of IFN-γ [18]. However, administration of TNF-α in vivo can actually decrease the severity and incidence of diabetes in NOD mice [29], and
anti-TNF-α antibody does not protect against the disease [30]. Moreover, RIP-TNF-α transgenic mice develop insulitis, but the inflammation does not progress to clinical diabetes [31]. Thus, TNF-α seems to function as an auxiliary in the pathogenic process, but not as a primary agent of disease.

In contrast to IFN-γ, IL-4 seems to have a protective effect on the disease. The administration of IL-4 has been reported to prevent the development of NOD diabetes [32]. Treatment of low-dose streptozotocin diabetes by anti-IFN-γ antibodies was associated with increased expression of IL-4 mRNA [27], and inhibition of autoimmune diabetes by the administration of complete Freund’s adjuvant or oral insulin was associated with a local increase in cells containing IL-4 [33, 34].

In this study, we adapted the ELISPOT assay to quantify the T cells capable, upon activation, of secreting these cytokines in the islets in various situations. The relatively small numbers of lymphocytes associated with the islets prevented us from testing T-cell responses to particular antigens. There was little or no spontaneous cytokine secretion by the islet T cells isolated in vitro, so we focused on the cytokines induced by activation with T-cell mitogenic anti-CD3 antibody. A notable advantage of the anti-CD3 activation was that our system was blind to cytokines produced by non-T cells and was free of bias for T cells specific for any particular antigen. Quantification of potential cytokine producers provided a global cytokine profile of the insulitis itself.

We found that the spontaneous development of IDDM (Figures 1 & 3) and its acceleration by cyclophosphamide (Figure 4) correlated with the accumulation of IFN-γ-secreting T cells in the islets. These observations support the conclusion that Th1 cells are important in the disease process. The secretion of TNF-α in the islets seems to be of ancillary importance, because TNF-α-producing T cells appeared at the same time in both males and females, although the females did show a greater number of such T cells (Figure 3). Moreover, the treatment of males with cyclophosphamide had a much less pronounced effect on the numbers of TNF-α secretors than on the numbers of IFN-γ secretors (Figure 4).

In view of the primary role of IFN-γ in the disease process, it is noteworthy that specific treatment with peptide p277 was associated with down-regulation of the numbers of IFN-γ-secreting T cells in the islets (Figure 5). This effect was specific in its induction: treatment with an immunogenic peptide from the mycobacterial variant of hsp60, MT-p278, did not inhibit the disease and did not significantly down-regulate the numbers of IFN-γ producing T cells (Figure 5). NOD mice in our colony show spontaneous T-cell proliferative reactivity and IFN-γ secretion to peptide MT-p278 [6], just as they do to p277 [3, 5]. Both peptides appear to feature the NOD MHC class II, I-A\(^{b}\) peptide binding motif and are comparably bound [35]. Thus, MT-p278 is a reasonable specificity control for p277 treatment.

Arrest of the development of diabetes by treatment with a single peptide, p277, is notable in view of the fact that a collective of diverse target antigens appears to be involved in the disease [2]. The effectiveness of p277 treatment was documented here by downregulation of the potential IFN-γ-secreting T cells in the islets responsive to anti-CD3 antibody. It is conceivable that anti-p277 T cells secreting a suppressor cytokine mediated a type of ‘bystander’ suppression [36] in the islets. However, such a ‘suppressor cytokine’ has yet to be characterized. We were able to detect IL-4 in the islets but could not demonstrate its upregulation, either because the period of IL-4 secretion is very brief, or because intraislet IL-4 may not be the p277-induced ‘downregulator’ of IFN-γ. Unfortunately, the ELISPOT assay has not yet been adapted by us to detect other ‘anti-inflammatory’ cytokines, such as IL-10 or TGF-β. Peptide p277
treatment was found to induce anti-p277 antibodies of the IgG2b isotype [5, 6], which is regulated by TGF-β [37]; so TGF-β could possibly be the elusive ‘down-regulator’. Improved assays could tell us this.

Interestingly, the p277 treatment did not influence the splenic T-cell cytokines that could be activated by the anti-CD3 mitogen. About three to four-fold more IFN-γ producers than IL-4 producers could be detected there irrespective of whether or not the mice had been treated with peptides p277 or MT-p278 (Figure 6B). Thus, although the administration of p277 seems to induce a shift in the specific anti-p277 T-cell population from a Th1 to a Th2 cytokine response [6], the peptide therapy does not change the systemic balance between Th1 and Th2 subpopulations.

The mechanisms by which the p277 peptide of human hsp60 protects NOD mice from IDDM are not clear. The human peptide differs from the mouse population from a Th1 to a Th2 cytokine response [6]. Thus, although p277 was altered in its sequence, we have no evidence to indicate that it acts as an ‘altered peptide ligand’ in a classical sense [39].

Note that arrest of insulitis by p277 treatment led to a relative decrease in the total numbers of islet-associated leukocytes and to a relative increase in the numbers of islets (Figure 7). It remains to be seen whether the apparent increase in islets was due to cessation of islet loss once the damage abated, to islet regeneration [40], or merely to greater efficiency in the isolation of islets that were relatively free of insulitis. In any case, the autoimmune process in murine type I diabetes is clearly susceptible to regulation by the positive induction of an alternate cytokine response to the peptide [6]. In the quest for therapy for autoimmune disease, it may be better to activate the immune system selectively, rather than try to deactivate it [7, 41].

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