Essential Role for Interferon-γ and Interleukin-6 in Autoimmune Insulin-dependent Diabetes in NOD/Wehi Mice

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Abstract

Experimental studies in vitro suggest that cytokines are important mediators in the pathogenesis of autoimmune insulin-dependent diabetes mellitus (IDDM). However, there is little evidence for the role of cytokines in vivo, either in humans or in the spontaneous animal models of IDDM such as the NOD mouse or BB rat. To address this question, we used the model of cyclophosphamide (CYP)-induced autoimmune diabetes in the NOD/Wehi mouse to examine for (*a*) the production of IFN-γ and IL-6 from isolated islets, and (*b*) the effect of anti IFN-γ or anti IL-6 monoclonal antibodies on the development of diabetes. After cyclophosphamide, the majority of these mice develop of mononuclear cell infiltrate (insulitis) which by 10–14 d is associated with beta cell destruction. IFN-γ activity at low levels (2.7±0.3 U/ml) could be detected only in culture supernatants from islets isolated at day 7 post-cyclophosphamide. In contrast, IL-6 activity progressively increased from 457±44 U/ml at day 0 to 6,020±777 U/ml at day 10. Culture of islets with anti-CD3 monoclonal antibody resulted in a significant increase in IFN-γ activity from 41±7 U/ml at day 0 to 812±156 U/ml at day 10. Mice given either anti-IFN-γ or anti-IL-6 antibody had a significantly reduced ($P < 0.001$) incidence of diabetes and especially with IFN-γ, decreased severity of insulitis. We conclude that IFN-γ and IL-6 have essential roles in the pathogenesis of pancreatic islet beta cell destruction in this model. (*J. Clin. Invest.* 1991. 87:739–742.) Key words: anti-cytokine therapy • pancreatic islet • beta cell

Introduction

A cellular autoimmune process that selectively destroys the pancreatic islet beta cells is thought to be responsible for the development of insulin-dependent diabetes mellitus (IDDM)[1] in humans (1, 2) and in the spontaneous animal models, the BB rat (3) and the NOD mouse (4). A common histopathologic feature associated with the development of IDDM is insulitis, the presence within and around the islets of mononuclear cells consisting predominantly of T lymphocytes and to a lesser degree macrophages (5–7). Experimental strategies aimed at suppressing cellular autoimmunity such as neonatal thymectomy, administration of cyclosporin A or administration of anti–T lymphocyte antibodies prevent development of diabetes (reviewed in references 8 and 9).

Although the molecular pathways for the initiation, perpetuation, and eventual destruction of the beta cell by autoreactive mononuclear cells are unknown, accumulating experimental evidence implicates cytokines as key mediators (reviewed in references 2 and 10). In addition to their actions on immunoinflammatory cells, the cytokines IFN-γ and TNF-α alone or in combination induce, in vitro, the expression of MHC-class I (11, 12), MHC-class II (13, 14) and ICAM-1 (15) molecules by murine and human beta cells. In addition IL-1 (16), IFN-γ, and TNF-α (17, 18) or combinations of these cytokines (17, 18) directly inhibit in vitro, the function and viability of beta cells.

Recently we found that beta cells produce IL-6 and that IL-6 mRNA and protein are markedly increased after exposure of murine islets to IFN-γ and/or TNF-α (19). In view of the wide ranging immunoregulatory effects of IL-6 (reviewed in reference 20), we have hypothesized that its production may confer on the beta cell accessory cell function and thereby perpetuate its immunologic destruction (19).

Apart from experimental studies in vitro there is little evidence that cytokines are present and/or play a role in the pathogenesis of autoimmune beta cell destruction. NOD/Wehi mice develop insulitis but have a low incidence of spontaneous diabetes. However, after a single injection of cyclophosphamide, insulitis is intensified and the majority of mice become diabetic within 2 wk (21). We have used this model to examine for (*a*) the production of IFN-γ and IL-6 from isolated islets, and (*b*) the effect of cytokine neutralization in vivo with anti-IFN-γ and anti-IL-6 antibodies on the development of diabetes.

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1. *Abbreviation used in this paper:* IDDM, insulin-dependent diabetes mellitus.

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Methods

Mice. Female NOD/Wehi mice, aged 70–80 d, were used in all experiments. Although insulitis is present in virtually all mice, the incidence of diabetes in female NOD/Wehi mice in contrast to most strains of NOD mice, is < 5% at age 100 d (21). There are no differences between NOD/Wehi and the high-incidence strain, NOD/Lt on the basis of reciprocal skin grafting and allelic enzyme analysis (22).

Monoclonal antibodies and cytokines. Monoclonal antibodies to IFN-γ (RA-642; hybridoma cells obtained from the American Type Culture Collection, Rockville, MD) and IL-6 (6B4; hybridoma cells kindly provided by Dr. J. Van Snick, Brussels, Belgium) were obtained from ascites produced in CBA-Nu/Nu mice. After partial purification

by NH_4SO_4 precipitation and dialysis, antibodies were diluted to a final concentration of 1 mg/ml in PBS and stored at −70°C until use. Monoclonal antibody to murine CD-3 (145-2C11; reference 23) was a gift from Dr. A. Kelso (Walter and Eliza Hall Institute of Medical Research). Rat immunoglobulin (whole Ig fraction; Jackson ImmunoResearch Labs, West Grove, PA) was diluted to 1 mg/ml in PBS before use.

Recombinant murine IFN-γ was a gift from Genentech Inc., South San Francisco, CA, and IL-6 from Dr. J. Van Snick, Brussels, Belgium. The specific activity of these cytokines was 2.3×10^7 U/mg and 1×10^8 U/mg, respectively.

Administration of cyclophosphamide. Euglycaemic mice were injected intraperitoneally on day 0 with 350 mg/kg cyclophosphamide (Cycloblastin, Farmitalia Carlo Erba, Hawthorn, Australia). At this dose some 40–60% of female NOD/Wehi became hyperglycaemic within 14 d.

Pancreatic islet isolation. For cytokine production experiments, islets were isolated by collagenase digestion from overnight fasted mice on days 0, 3, 7, and 10 after cyclophosphamide. Briefly, each pancreas was distended with 10–15 ml of sterile Hepes buffered Kreb's-Ringer bicarbonate buffer, pH 7.5, containing 1 mg/ml BSA and 1 mg/ml D-glucose (HKRB), cut into eight pieces and subsequently placed in sterile vials containing 6 ml of HKRB buffer with 0.8 mg/ml collagenase (type XI; Sigma Chemical Co., St. Louis, MO). After shaking (300 rpm) in an orbital shaker for 25 min at 37°C, digested tissue was washed twice with 10 ml of HKRB buffer, filtered through a sterile 500 μm screen and pelleted by gentle centrifugation (800 $g \times 1$ min). Isolated islets were then purified by discontinuous density-gradient centrifugation through Ficoll (Ficoll-400; Sigma Chemical Co.) at densities of 1.085, 1.075, 1.065, 1.055. Islets present at the 1.075/1.065 and 1.065/1.055 boundaries were further purified by handpicking with the aid of a dissection microscope. This procedure permits the isolation of islets and the associated insulitis lesion, the latter being visible as a clear crescent-shaped mantle surrounding the islet. Groups of 150–200 islets were placed into individual wells of a 24-well cluster plate (Costar, Cambridge, MA) containing 750 μl RPMI-1640 supplemented with 5×10^{-5} M 2-mercaptoethanol, antibiotics, and 10% heat-inactivated FCS (CSL, Parkville, Australia) (RPMI-FCS) and incubated for 72 h. Islets prepared as described above were also cultured in 24-well plates previously coated with CD-3 monoclonal antibody. Purified antibody at 5 μg/ml in PBS was incubated in the wells for 6 h at 37°C, unbound antibody was removed by washing three times in PBS, and coated plates stored at 4°C until use.

Cytokine assays. Assays for IL-6 and IFN-γ were performed using the IL-6 dependent murine plasmacytoma TEPC-2027 (24) and the IFN-γ-sensitive Wehi-279.1 cell lines. TEPC or Wehi cells were seeded in 96-well microtiter plates in RPMI-FCS and cultured at 10^4 cells per well (final volume, 100 μl) in the presence of serial dilutions of culture supernatants or recombinant standard. At day 3 the number of viable cells was measured with the 3-(4,5-dimethylthiazol-2-*yl*)-2,5-diphenyl tetrazolium bromide method described by Tada et al. (25) modified as described previously (19). The minimal detection limit was ~ 2 and 5 U/ml for IFN-γ and IL-6, respectively.

Administration of anticytokine antibodies. Euglycemic mice were randomly allocated into three groups of 20 to receive rat Ig, RA-642 or 6B4 in a dose of 0.5 ml (~ 500 μg) on days −1, 3, 6, 9, and 12. Blood samples were collected by orbital sinus bleeding. Blood glucose was measured immediately with B-M glucose test strips (Boehringer-Mannheim, Melbourne, Australia). Mice were considered diabetic when blood glucose was > 15 mmol/liter.

Quantitation of insulitis. Insulitis was scored from the histological appearance of at least 10 islets in a minimum of three sections of each pancreas removed at day 15 and fixed in Bouin's solution. Each islet was scored as to the degree of mononuclear cell infiltration as follows: 0, no infiltration; 1, periductular infiltrate; 2, periislet and periductular infiltrate; 3, intraislet infiltrate; 4, intraislet infiltrate associated with beta cell destruction. The mean score for each pancreas was calculated by dividing the total score by the number of islets scored.

Results

Production of IL-6 and IFN-γ from isolated islets. Supernatants from cultures of NOD islets isolated serially after cyclophosphamide were analyzed for IFN-γ and IL-6 activity (Table I). Low level IFN-γ activity could be detected in culture supernatants from day 7 islets only. In contrast, significant levels of IL-6 activity increased progressively in culture supernatants from islets isolated at day 0 through day 10.

To determine whether or not the low IFN-γ production was due to the absence of functional T cells, isolated islets were cocultured with solid-phase anti-CD3 antibody (Table II). Under these conditions significant IFN-γ activity was detected in the islet supernatants which, in comparison with day 0, decreased at day 3 before increasing significantly at day 7 and 10. Addition of RA-642 antibody completely abolished the IFN-γ activity in day 10 supernatants. Increased IL-6 activity was also present in day 0 and 3 supernatants and further increased to the same level in day 7 and 10 supernatants (Table II). Addition of 6B4 antibody almost completely abolished the IL-6 activity in day 10 supernatants.

Effect of anticytokine antibodies on the development of diabetes. On day 15 after cyclophosphamide administration, 9 of 19 (47%) control mice were diabetic compared with only 1 of 20 (5%) mice treated with anti-IFN-γ antibody and 3 of 19 (16%) mice treated with anti-IL-6 antibody (Table III).

Examination of the pancreas revealed a reduction in the severity of insulitis in mice treated with anticytokine antibody, especially anti-IFN-γ compared with the control mice (Table III).

Discussion

Apart from numerous experimental studies in vitro there has been little direct evidence for the role of cytokines in the pathogenesis of IDDM, either in humans or in the spontaneous animal models. In the present study we used cyclophosphamide-induced autoimmune diabetes in the NOD/Wehi mouse as a model in an attempt to provide this evidence. First, we looked for the production of the cytokines IFN-γ and IL-6 from isolated islets, and second, we examined the therapeutic potential of injected anti-IFN-γ and anti-IL-6 antibodies. The cyclophosphamide model has particular advantages in that the process of autoimmune beta cell destruction is synchronized and the time to onset of hyperglycemia considerably shortened (21). Furthermore cyclophosphamide has been reported not to

Table I. IFN-γ and IL-6 Production from Islets Isolated from Cyclophosphamide-injected NOD/Wehi Mice

Cytokine	Cytokine activity Day after cyclophosphamide injection			
	0	3	7	10
	U/ml			
IFN-γ	ND	ND	2.7±0.3	ND
IL-6	457±44	1136±325	2933±548*	6021±777*

Results are expressed as mean±SEM for triplicate cultures.
ND, not detectable.
* For significance vs. day 0, $P \leq 0.05$ (two-tail Student's t test).

Table II. IFN-γ and IL-6 Production from NOD/Wehi Islets Cultured with Anti-CD3 Antibody

	Cytokine activity Day after cyclophosphamide injection					
					10	
Cytokine	0	3	7	10	+RA-642	+6B4
			U/ml			
IFN-γ	41±13	17±4	378±63*	812±156*	ND	635±92
IL-6	3202±725	2950±700	6188±738*	5494±568*	5386±470	48±9

Results are expressed as mean±SEM for triplicate cultures. ND, not detectable. * Significance vs. day 0, $P < 0.05$ (two-tail Student's t test).

influence beta cell function or viability (26) suggesting that the drug is not directly beta cell toxic. The mechanisms of beta cell destruction in this model are clearly autoimmune and cannot be distinguished from that occurring spontaneously in NOD mice (21, 26). In addition the numbers and phenotype of T lymphocytes infiltrating the pancreas has been defined in this model (27). Therefore, the use of the collagenase digestion procedure which permits the isolation of the intact islet-insulitis lesion makes it possible to study islet-associated T lymphocytes at strategic time points before or after administration of cyclophosphamide.

In the studies with isolated islets barely detectable levels of IFN-γ activity were present and only in supernatants from islets at day 7 postcyclophosphamide. It is not known whether the low level of IFN-γ activity reflects that in vivo in the extracellular milieu or whether T lymphocytes producing IFN-γ are downregulated upon exposure to in vitro culture conditions. Clearly islet-associated T lymphocytes have the potential to produce larger amounts of IFN-γ as evidenced by the results of the experiments in which T lymphocytes were activated via the T cell receptor pathway using an anti-CD3 antibody. In parallel experiments using purified pancreatic T lymphocytes we have observed a qualitatively similar result with significant production of IFN-γ being observed only after stimulation with anti-CD3 antibody (Kay, T., unpublished observation). The IFN-γ activity produced from the unstimulated islet T lymphocytes, although lower than that required in vitro to inhibit beta cell function and viability (17, 18) is within the range for upregulation of beta cell MHC-class I molecules (11) and is therefore biologically relevant.

In contrast to IFN-γ, IL-6 activity was significantly elevated in culture supernatants from islets isolated before cyclophosphamide injection and progressively increased thereafter, indicating a temporal association between production of this cytokine and evolution of the autoimmune lesion. Elevated production of IL-6 has been demonstrated in a number of autoimmune diseases (28). For example, high levels of IL-6 have been found in the synovial fluid of patients with active rheumatoid arthritis (29) and an association between synovial fluid IL-6 and disease activity was reported in patients with inflammatory arthritis of traumatic arthritis (30). The present findings also support the view that the local production of IL-6 reflects disease activity. The fact that stimulated IL-6 production appeared to plateau by day 7 may indicate that a major source of IL-6 is CD4-positive T lymphocytes that predominate at this stage (27). We have reported that beta cells also produce IL-6 and IFN-γ and TNF-α significantly enhance this IL-6 production (19). As has previously been suggested (31) the production of IL-6 induced by cytotoxic cytokines such as IFN-γ and TNF-α or as seen in the present study may constitute part of an injury response by the beta cell that could serve to further enhance the inflammatory response.

Table III. Diabetes Incidence and Degree of Insulitis in Cyclophosphamide-treated NOD/Wehi Mice Given Monoclonal Antibody to Either IFN-γ or IL-6

Antibody	Diabetes, day 15	Insulitis score
Rat Ig (control)	9/19	2.60±0.34
RA-642 (anti-IFN-γ)	1/20*	1.25±0.25‡
6B4 (anti-IL-6)	3/19*	2.10±0.34

* For significance vs. control $P < 0.01$ and $P < 0.05$ (χ^2 test) for HB-170 and 6B4, respectively.
‡ Mean±SEM: significance vs. control, $P < 0.001$ (two-tail Student's t test).

Neutralization of IFN-γ activity in vivo resulted in a pronounced decrease in the incidence of diabetes and was associated with a significant reduction in severity of insulitis, suggesting that anti-IFN-γ treatment is preventative for disease. In contrast to IFN-γ, neutralization of IL-6 activity in vivo while resulting in a reduction in the incidence of diabetes at day 15 was not associated with a significant reduction in insulitis. It is therefore not possible to conclude from the present study whether anti-IL-6 treatment is preventative or delays the onset of diabetes. These differences between IFN-γ and IL-6 may simply reflect the more efficient neutralization of endogenous IFN-γ versus IL-6. Alternatively, IFN-γ may be the more critical cytokine in the initial development of insulitis while IL-6 might be required for subsequent perpetuation, e.g., through enhanced cytotoxic T lymphocyte function (32) of autoimmune beta cell destruction.

In view of their multiple overlapping and cross-regulatory functions (33), it is reasonable to assume that other cytokines, in addition to IFN-γ and IL-6, have a role in the pathogenesis of autoimmune beta cell destruction. As discussed earlier, based on studies in vitro, IL-1 and TNF-α would be good candidates in this role. To our surprise, we have not been able to detect either of these cytokines in supernatants from islets cultured with or without anti-CD3 antibody at any of the time points after cyclophosphamide injection in NOD/Wehi mice (Campbell, I. L., unpublished). In accord with our finding is the recent demonstration that in comparison with SWR mice, peritoneal exudate cells from NOD (high diabetes incidence

strain) mice produce only low levels of TNF-α and IL-1β (34). Therefore the pattern of cytokine expression associated with the development of autoimmune beta cell destruction in the NOD mouse appears to be complex ranging from enhanced production of IFN-γ and IL-6 on the one hand, to deficient production TNF-α and IL-1α on the other.

In conclusion, evidence has been obtained in vivo for an essential role for the cytokines IFN-γ and IL-6 in autoimmune beta cell destruction. Further studies should be directed at defining a composite picture of cytokine expression, and the cellular sources of specific cytokines, during the evolution of insulitis. Our findings are an impetus to the development of anticytokine therapies to prevent beta cell destruction in humans with preclinical IDDM.

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