Treatment With Neutralizing Antibodies Specific for IL-1β Prevents Cyclophosphamide-Induced Diabetes in Nonobese Diabetic Mice

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Interleukin-1 (IL-1) has been shown to be involved in the pathogenesis of IDDM, but it is not clear which form, IL-1α or IL-1β, is predominantly implicated. In this study, we have evaluated the contribution of IL-1β by treating diabetes-prone nonobese diabetic (NOD) mice with specific neutralizing antibodies. First, we assessed the neutralizing potential of these antibodies in C57BL/6 mice under acute septic shock by measuring IL-1β in sera 4 h after lipopolysaccharide injection. One milligram and 0.1 mg of anti–IL-1β antibodies (Abs) were capable of neutralizing the IL-1β produced, and the effect persisted for at least 5 days. Second, we evaluated the role of IL-1β in the cyclophosphamide (CY)-accelerated model of diabetes. Nondiabetic male NOD mice were injected with 200 mg/kg CY and treated twice weekly with anti–IL-1β Ab. The incidence of diabetes reached 76 and 100% in the control groups treated with 0.25 and 0.1 mg rabbit IgG, respectively. In contrast, only 34% of mice treated with 0.25 mg of anti–IL-1β Ab became diabetic. In the group treated with 0.1 mg of anti–IL-1β Ab, 89% of the mice became diabetic in the same period of time, demonstrating that the protective effect was dose dependent. Our results show that IL-1β is a critical effector molecule in this model of IDDM and that its specific inhibition could be an attractive target for therapeutic intervention. ***Diabetes*** **46:937–940, 1997**

Interleukin-1 (IL-1) is a pleiotropic multifunctional cytokine implicated in a vast array of biological responses (1). Through its pro-inflammatory properties, IL-1 is thought to play an active role in the pathogenesis of autoimmune diseases, including IDDM (2). The implication of IL-1 in the selective destruction of insulin-producing β-cells is supported by a large body of experimental evidence (3). In vitro, IL-1 causes severe structural and functional alterations of islet β-cells, presumably mediated through nitric oxide (4). In vivo, the neutralization of endogenously produced IL-1 by administration of the natural receptor antagonist (IL-1 ra) or a genetically engineered soluble receptor (sIL-1R) prevents IDDM onset in BB rats (5) and nonobese diabetic (NOD) mice (6).

There are two forms of IL-1, IL-1α and IL-1β (7). Both bind with high affinity to the same receptors and exert parallel pro-inflammatory responses. They differ, however, in their synthesis pathways and their distribution. In humans particularly, IL-1α remains within the cells or anchored to the surface of cells such as macrophages or monocytes, whereas IL-1β is preferentially released as a free hormone-like mediator (8,1). Moreover, in contrast to IL-1α, which is active in its synthesized form, IL-1β is synthesized as a 31-kDa inactive precursor that becomes bioactive upon proteolytic cleavage by a cysteine protease, the IL-1–converting enzyme (ICE) (9,10). Mice made deficient for ICE by gene targeting and homologous recombination are unable to produce mature IL-1β and acquire resistance to lipopolysaccharide (LPS)-induced septic shock (11,12).

It is still not clear whether one form of IL-1 is implicated more than the other in the pathogenesis of IDDM. Even though IL-1β is toxic in vitro against islet β-cells (13), it has not been demonstrated that it acts similarly in vivo. The injection of either IL-1α or IL-1β into NOD mice (14) or into BB rats (15) has provided ambiguous results with regard to their respective pathogenicity, and experiments using receptor antagonists or competitors have not been informative on this specific issue because both forms of IL-1 are antagonized. Because IL-1 represents a major therapeutic target for IDDM, we decided to evaluate the precise contribution of one of the two forms, the β form, by treating NOD mice with specific IL-1β–neutralizing antibodies (Abs). We chose the cyclophosphamide (CY)-accelerated model, which is IL-1 dependent and responds to sIL-1R competitors (6). Our results show that the disease can be prevented in a majority of mice treated with anti–IL-1β Abs, thus indicating that IL-1β is a critical effector molecule in this model of IDDM and that strategies aimed at its specific inhibition may have therapeutic potential.

RESEARCH DESIGN AND METHODS

Mice. C57BL/6J and NOD/Nck mice were raised and maintained in our facility under specific pathogen–free conditions.

Antibodies and cytokines. Polyclonal anti-murine IL-1β Abs were prepared in rabbits according to a protocol of immunization adapted from Högquist et al. (16). Rabbits were primed subcutaneously in multiple sites with 20 μg of highly purified recombinant mouse IL-1β (Euromedex, Souffelweyersheim, France) adsorbed on aluminum hydroxide. They were subsequently boosted with 10 μg of cytokine plus alum at days 14, 28, 56, and 84. At the same time, the rabbits were given an emulsification of saline in complete Freund's adjuvant at sites adjacent to those of antigen application. Animals were bled via the ear vein at days 38, 66,

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Ab, antibody; CY, cyclophosphamide; ICE, IL-1–converting enzyme; IFN-γ, γ-interferon; IL-1, interleukin-1; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; TNF-α, tumor necrosis factor-α.

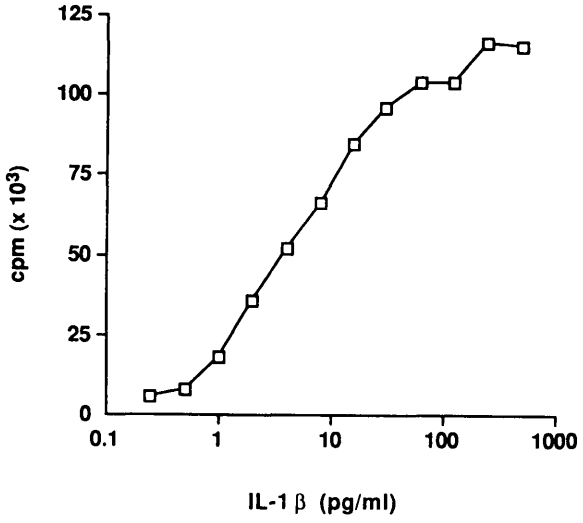

FIG. 1. Standard curve of recombinant IL-1β in the bioassay D10G4.

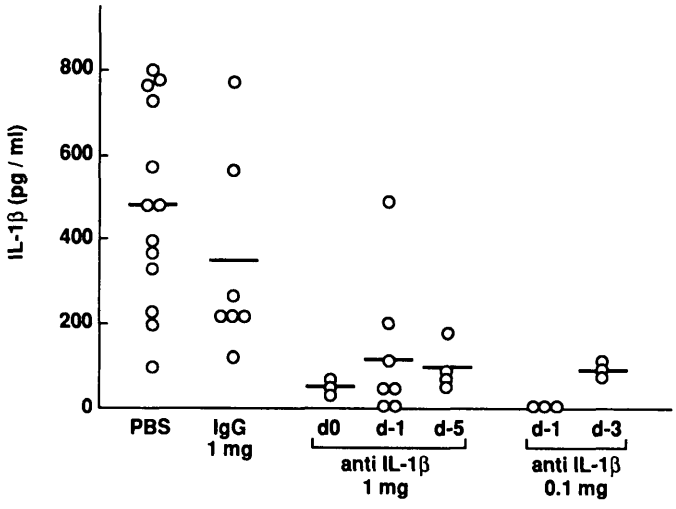

FIG. 2. Effect of anti-IL-1β Ab treatment on IL-1β production in murine septic shock. C57BL/6J mice were injected with 300 μg LPS and treated with 1 or 0.1 mg anti-IL-1β Ab, either 2 h after LPS injection (d 0) or 1 to 5 days before LPS shock (d-1, d-3, d-5). Control groups were treated with PBS or 1 mg rabbit IgG. IL-1β levels were measured in the sera taken 4 h after LPS injection in the biological assay using the IL-1–dependent T-cell clone D10G4.

and 94. Final bleeding was taken at day 122. Antibodies were purified by ammonium sulfate precipitation of immune sera and protein G sepharose 4 fast flow chromatography (Pharmacia Biotech, Orsay, France). Control IgG was purified in the same way from normal rabbit serum.

Murine IL-1α, murine IL-1β, and anti-murine IL-1α neutralizing Abs used in the titration assays were purchased from R&D (Abingdon, U.K.).

Induction of endotoxinic shock and biological assay for IL-1β. Septic shock was induced in 10- to 12-week-old C57BL/6J mice by an intraperitoneal injection of 300 μg/mouse of LPS from *Escherichia coli* serotype 0111:B4 (Sigma, Saint Quentin Fallavier, France). Sera from control and Ab-treated mice were collected 4 h later.

The biological activity of IL-1β released in the serum was evaluated in an assay measuring the IL-1–dependent co-stimulation of the T-cell clone D10G4, according to Kaye et al. (17). D10G4 T-cells (1.5×10^4), freshly passaged in complete RPMI 1640 medium (GIBCO BRL, Cergy Pontoise, France) plus 10% fetal calf serum (Techgen, Les Ulis, France), were co-stimulated in 96 multiwell plates with 8 μg/ml Concanavalin A (MilesYeda, Rehovoth, Israel) and serial dilutions of sera. To make the assay specific for IL-1β, 5 μg/ml of anti-IL-1α Ab was added to the serum 1 h before the addition of the D10G4 cells and was left for the whole assay. After 3 days of culture, DNA synthesis was measured by [^{3}H]thymidine incorporation for 6 h. An example of a standard curve done with recombinant IL-1β is shown in Fig. 1. The sensitivity of the assay is in the range of 1 pg/ml. IL-1α concentrations were calculated by subtraction of IL-1β concentrations from total IL-1.

Induction of overt diabetes by CY and experimental design. Nondiabetic 7-week-old NOD males were injected with a single dose of CY (Sigma) at 200 mg/kg. The mice were then randomly divided into two groups. The experimental group was treated twice weekly for 2 weeks, starting on the day of CY injection, with either 0.25 mg or 0.1 mg of anti-IL-1β Ab. The control group received either 0.25 mg or 0.1 mg of control rabbit IgG. Each mouse thus received four injections of Ab. Mice were monitored three times a week for the onset of glycosuria and were considered as overtly diabetic on the basis of two consecutive positive glycosuria tests (Glukotest test strips, Boehringer Mannheim, Meylan, France) and blood glucose level >20 mmol/l. Glycemia was measured by colorimetric quantitative assay (Haemoglokotest and Reflolux F, Boehringer Mannheim).

Statistical analysis. Statistical analyses were performed using the Mann-Whitney *U* test for dosage of IL-1β and the χ^2 test for diabetes incidence.

RESULTS

In vivo activity of anti-IL-1β Ab in mice undergoing endotoxinic shock. We first assessed the neutralizing potential of the anti-IL-1β Ab in vivo in mice undergoing septic shock induced by LPS. We also verified that the Abs were specific for IL-1β and that their effects lasted for at least several days. C57BL/6J mice were injected intraperitoneally with 300 μg LPS, a dose shown to induce 4 h later a sharp burst of IL-1 (C. Gardner, Roussel-Uclaf, personal communication). Therapeutic Abs were administered as a single intraperitoneal injection of 0.1 or 1 mg IgG, either 2 h after LPS injection to test their neutralizing potential at the peak of IL-1 release or 1 to 5 days before LPS shock to evaluate their efficacy after several days in the recipient. Control mice received phosphate-buffered saline (PBS) or normal rabbit IgG following the same regimen. The results, summarized in Fig. 2, show that in spite of a certain heterogeneity of the IL-1 response to LPS challenge, anti-IL-1β Abs were capable of neutralizing the burst of IL-1β in most animals. The differences in circulating IL-1β concentrations between the IgG control group and the various Ab-treated groups were statistically significant ($P < 0.05$ by the Mann-Whitney *U* test). The two doses of Ab were practically equivalent and the effect clearly persisted for at least 5 days. As can be seen from Table 1, where IL-1α was titrated, the administration of anti-IL-1β Ab did not affect IL-1α production and release. It should be noted that at variance with human IL-1α, murine IL-1α is more readily released into the circulation (1,8). If anything, the titers in treated mice were slightly augmented compared with the controls. In view of these results, we decided to treat NOD mice twice weekly with doses of 0.25 and 0.1 mg IgG.

Prevention of diabetes in CY-induced NOD mice treated with anti-IL-1β Ab. CY at doses of 200–300 mg/kg precipitates the onset of IDDM in NOD mice. The accelerated form of the disease is similar in many respects to the natural one. It is T-cell and macrophage dependent (18); it can be adoptively transferred by T-cells (19); and for its expression it requires the same set of *Idd* genes as those required in the spontaneous form of the disease (20). As shown in Fig. 3, which summarizes the results of three independent experiments, a single dose of 200 mg/kg CY caused a rapid appearance of overt diabetes in young NOD males. The incidence reached 76% (22/29) and 100% (10/10) in the 0.25 and 0.1 mg IgG control groups, respectively, and remained stable up to 1 month after CY injection. The incidence in PBS-treated controls was similar, indicating that rabbit IgG per se did not

TABLE 1
Anti–IL-1β Ab treatment does not affect IL-1α production

Treatment	IL-1α (pg/ml)
PBS	482 ± 77
IgG control	406 ± 148
Anti–IL-1β (day 1)	667 ± 133
Anti–IL-1β (day 5)	554 ± 128

Data are means ± SE of four sera from individual mice. Mice were treated with 1 mg anti–IL-1β.

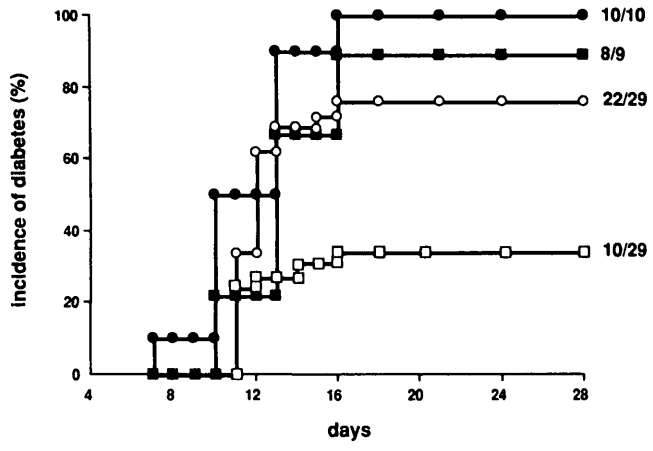

FIG. 3. Effect of anti–IL-1β Ab treatment on CY-induced diabetes in NOD mice. Mice were injected with 200 mg/kg CY and treated twice a week for 2 weeks, with 0.25 mg anti–IL-1β Ab (□) or rabbit IgG control (○), or with 0.1 mg anti-IL-1β Ab (■) or IgG control (●). Difference in cumulative incidence between the experimental group treated with 0.25 mg anti–IL-1β Ab and the control group injected with the same amount of IgG is statistically significant at $P < 0.01$ by χ^2 test.

influence disease evolution (data not shown). In contrast, only 34% (10/29) of mice treated with 0.25 mg anti–IL-1β Ab became overtly diabetic during the same period of time. The difference is significant at a P value <0.01 by χ^2 test. The protective effect was clearly dose dependent, since NOD mice treated with 0.1 mg displayed an incidence of diabetes comparable to that of the controls (89%, 8/9).

Histology performed on pancreatic samples from treated and control mice demonstrated definite alterations, including intra-islet lymphocyte infiltration and islet atrophy. Severity was not significantly reduced in the anti–IL-1β Ab treated mice (data not shown).

DISCUSSION

This report shows that antibodies specifically neutralizing IL-1β have a protective effect on CY-induced IDDM in NOD mice. It therefore confirms the results of Nicoletti et al. (6) showing that neutralization of IL-1 by sIL-1R reduced IDDM incidence under the same conditions. The present results demonstrate, in addition, the IL-1β dependency of the pathological process, information that could not be inferred from the previous study. The treatment with anti–IL-1β Ab does not simply delay the onset of hyperglycemia but durably prevents its occurrence over a period of 1 month after induction. Neither is the treatment masking the presence of the disease by artificially lowering blood glucose. The fact that protected mice are normoglycemic at values well below 12–15 mmol/l and that blood glucose in those mice remains stable after treatment cessation argues against such a putative extrapancreatic effect of anti–IL-1β Ab.

The fact that insulitis is not significantly attenuated in those mice is in line with the report of Nicoletti et al. (6), which showed similar resistance of insulitis to treatment with soluble IL-1 receptor. These results are also consistent with the view that in vivo neutralization of IL-1β should prevent the end-stage process, namely macrophage activation and β-cell injury, but should presumably not inhibit islet invasion by T-cells. Whether IL-1β neutralization will similarly prevent the end-stage process of the natural disease remains an open question. Long-term treatment with rabbit anti–IL-1β Ab has not been presently considered because the mice become rapidly and ineluctably immune against the heterologous immunoglobulins.

The protection afforded by anti–IL-1β Ab is clearly dose dependent. The fact that 0.1 mg of Ab was unable to prevent CY-induced diabetes in NOD mice whereas it neutralized the LPS-induced burst of IL-1β in C57BL/6 mice suggests two observations. The first one is that it is probably necessary to maintain sustained neutralization of IL-1β production to prevent the destruction of the islets. Second, it is possible that the NOD strain produces, under special circumstances such as the injection of a sublethal dose of CY, exaggerated amounts of IL-1β. Although IL-1β production by NOD peritoneal macrophages pulsed in vitro with LPS is decreased, as reported by Serreze and Leiter (21), NOD mice challenged in vivo with *Propionibacter acnis* and LPS have been found to produce 10 times more IL-1β than BALB/c and C57BL/6 controls (C. Gardner, S. Spinella-Jaegle, personal communication).

Other pro-inflammatory cytokines, including IL-1α itself, may be implicated in experimental IDDM. The small number of mice escaping protection in the treated group could be explained by the presence of normal levels of IL-1α. In addition, we and others have reported that anti–γ-interferon (IFN-γ) Abs could prevent CY-induced disease and adoptive transfer by diabetogenic T-lymphocytes in NOD mice (22,23). Similar results have been reported with IL-6, another pro-inflammatory cytokine (23). The implication of tumor necrosis factor-α (TNF-α) in IDDM pathogenesis has been demonstrated in several experimental systems: in transgenic mice carrying the TNF-α gene under a rat insulin promoter (24, 25), in young NOD mice treated with TNF-α at very early onset of the autoimmune process (26), and more recently in NOD mice with a transgene encoding for a soluble form of the p55 TNF receptor (26a). In all these models, TNF-α was found to promote IDDM while antagonists prevented the process. A dramatic acceleration of the disease has also been reported in prediabetic NOD mice receiving IL-12 (27). Altogether, these results raise the question of the specificity of each of these inflammatory cytokines and of the role of other putative effector agents, such as cytotoxic CD8 T-cells, in the pathogenesis of IDDM (29). It is indeed difficult to understand how the autoimmune process leading to overt diabetes can be prevented by blocking a single one of these agents. The specificity of the treatments cannot be questioned. As we show here in animals treated with anti–IL-1β Abs, the production and release of IL-1α remains normal. Similarly, treat-

ment of NOD mice with sIL-1R preserves their capacity to produce IL-2 or IFN-γ (6). It is conceivable that each cytokine provides its own specific and unsubstitutable contribution to β-cell destruction so that by neutralizing a single cytokine, the whole process is inhibited. It seems more likely, however, that cytokines act in a synergistic and coordinated fashion. Blocking one of them may have an important impact on the production of the others and on the whole pathological process leading to overt diabetes. The fact that IL-1 or TNF-α may precipitate or delay IDDM onset depending on the site of cytokine production, its concentration, and the age and the genetic background of the mice (14,15,26,29) probably reflects the complexity of cytokine involvement in autoimmune disorders.

In any event, this study suggests that IL-1β is an attractive target for immunointervention. The possibility of specifically controlling the production of this cytokine via the inhibition of ICE opens interesting perspectives for treatment of IDDM and probably other autoimmune disorders. Future effort will have to be invested in the design of efficient and long-lasting inhibitors of ICE while keeping in mind that this enzyme exerts other important biological functions related to apoptosis and the homeostasis of the immune system (30).

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