Recombinant Human Tumor Necrosis Factor α Suppresses Autoimmune Diabetes in Nonobese Diabetic Mice

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Abstract

We previously reported that administration of a streptococcal preparation (OK-432) inhibited insulitis and development of autoimmune diabetes in nonobese diabetic (NOD) mice and BB rats as animal models of insulin-dependent diabetes mellitus. In this study, we screened various cytokines that could be induced by OK-432 in vivo, for their preventive effect against diabetes in NOD mice. Among recombinant mouse IFNγ, human IL1α, human IL2, mouse granulocyte-macrophage colony-stimulating factor and human TNFα, only human TNFα suppressed insulitis and significantly ($P < 0.001$) inhibited development of diabetes. NOD mice were the lowest producers of the mRNA of TNF and serum TNF on stimulation with OK-432 or with IFNγ plus LPS, compared with C57BL/6, C3H/He, and Balb/c mice. The results imply a role for low productivity of TNF in the pathogenesis of autoimmune diabetes in NOD mice.

Introduction

Diabetes develops spontaneously in the nonobese diabetic (NOD)[1] mouse (1), an animal model of insulin-dependent diabetes mellitus (IDDM),[1] as a result of pancreatic B cell destruction by autoimmunity (2). The trigger of induction of antiislet autoimmunity and the mechanism of persistent progression of the autoimmunity in NOD mice are not understood, although it has been reported that the development of diabetes was genetically controlled by at least two or three recessive genes (3–5), and that the effector cells for B cell destruction were T lymphocytes (6–9). Various immune interventions have prevented development of autoimmune diabetes in NOD mice: e.g., suppression of cellular immunity by administration of anti thymocyte antibody (10), anti-L3T4 antibody (6, 7), cyclosporin A (11), silica (12), elimination of immunocompetent cells with irradiation followed by allogeneic bone marrow transplantation (13), and transgene of the I-Eα chain, which is lacking in NOD mice (14). In addition, we previously reported that immunomodulation by a streptococcal preparation (OK-432) inhibited insulitis and development of IDDM in NOD mice (15) and BB rats (16), and that OK-432 treatment suppressed generation of effector cells for B cell destruction (Shintani, S., J. Satoh, H. Seino, Y. Goto, and T. Toyota, manuscript submitted for publication).

OK-432 is one of a number of potent biological response modifiers that are used clinically for cancer immunotherapy (17) and are known to induce various cytokines (18, 19). To clarify the role of cytokines in the inhibition of diabetes by OK-432, we screened recombinant lymphokines and monokines of human or mouse origin for their possible preventive effects in diabetes of NOD mice. We found that recombinant human tumor necrosis factor α (hTNFα) suppressed the development of diabetes in NOD mice and that the NOD mouse strain was a low producer of endogenous TNF.

Methods

Mice. NOD(H-2K^d, I-A^{NOD}, D^b), C57BL/6(H-2^b), C3H/He(H-2^k) and Balb/c(H-2^d) mice were maintained in specific pathogen-free conditions at the Institute for Experimental Animals, Tohoku University School of Medicine. Only female mice were used for the experiment because of the high incidence of diabetes in female NOD mice.

Source and administration of OK-432 and cytokines. OK-432 and each of the recombinant cytokines were kindly provided by the following pharmaceutical companies: OK-432 by Chugai Pharmaceutical Co. (Tokyo, Japan), mouse (m) IFNγ and human (h) IL2 by Shionogi Pharmaceutical Co. (Osaka, Japan), mouse granulocyte-macrophage colony-stimulating factor (mGM-CSF) by Sumitomo Pharmaceutical Co. (Tokyo, Japan), hIL1α by Dainippon Pharmaceutical Co. (Osaka, Japan) and hTNFα by Asahi Chemical Industrial Co. (Tokyo, Japan). The dose of OK-432 or each of the recombinant cytokines was administered to mice according to the schedule indicated in Table I.

Definition of diabetes. Urine glucose was tested with a Tes-tape (Eli Lilly & Co., Indianapolis, IN) twice a week. Mice were defined as diabetic when they showed persistent glycosuria greater than (+) with a Tes-tape or their nonfasting blood glucose concentrations were > 200 mg/dl.

Histology. Pancreata of the NOD mice were fixed with 10% formaldehyde solution, mounted with paraffin, cut to 3-μm sections, stained with hematoxylin and eosin solution, and then observed by light microscopy.

Assay of TNF activity. TNF activities in sera were assayed by using LM cells, a subline of TNF-sensitive mouse fibroblast (L929), as a target cell and by using recombinant human TNFα as a standard. Briefly, 2 × 10^5 LM cells suspended in 0.2 ml of RPMI 1640 medium

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1. *Abbreviations used in this paper:* IDDM, insulin-dependent diabetes mellitus; NOD, nonobese diabetic; PEC, peritoneal exudative cells; TNF, tumor necrosis factor.

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supplemented with 5% fetal bovine serum were cultured in a 96-well microculture plate. After confluent cell growth, the medium was replaced with twofold diluted mouse serum containing 1 μg/ml of Actinomycin D. For neutralization of mouse serum TNF, serum was preincubated overnight with rabbit anti-mouse TNF antibody (kindly provided by Y. Terano, Suntory Co., Osaka, Japan). 24 h later, 100 μl of 0.2% crystal violet was added to each well and incubated for 3 min to stain the cells. After gentle and extensive washing in water, the plates were dried and OD at 540 nm was measured with a Multiskan (Titertek; Flow Laboratories, McLean, VA).

Northern blot analysis of TNF mRNA. Mice were injected intraperitoneally with 0.1 mg of OK-432 at 1-wk intervals. 3 h after the second injection, peritoneal exudative cells (PEC) were collected and RNA was extracted from the PEC and purified by centrifugation through a CsCl gradient (20). 10 μg of isolated, total RNA were size-fractionated by electrophoresis through a 1.2% agarose gel, and transferred to a nylon membrane. Hybridization was performed with ^{32}P-labeled cDNA of mTNF (kindly provided by Asahi Chemical Industrial Co.) (21).

Results

Effect of various cytokines on the development of diabetes. Recombinant human or mouse cytokines were administered twice a week to NOD mice 4–25 wk of age, and the cumulative incidence of diabetes was compared to that of nontreated control mice. OK-432 completely prevented the development of diabetes in NOD mice (Table I) as previously reported (15). As shown in Table I, mIFNγ and hIL2 had neither suppressive nor promotive effects on the incidence of diabetes. However, hIL2 delayed the time of onset of overt diabetes by 2–4 wk, compared with that of nontreated mice (data not shown). mGM-CSF and hIL1α lowered the incidence of diabetes, although the difference was not significant. Among the various cytokines tested, only hTNFα significantly ($P < 0.001$) inhibited the incidence of diabetes in NOD mice.

Pancreas histology in hTNFα-treated NOD mice. The NOD mice were killed at 20 wk of age and pancreas sections were stained with hematoxylin-eosin and observed by light microscopy. Because we could find few islets in the pancreas section from diabetic mice because of the destruction of B cells, most of the islets counted were in the sections from nondiabetic mice.

Table II shows that insulitis was more intense in the nontreated mice. On the other hand, hTNFα reduced the degree of insulitis compared with nontreated mice, although the suppressive effect of hTNFα was not as strong as that with OK-432.

Production of endogenous TNF in various strains of mice. To assess the endogenous TNF production capability in vivo, various strains of mice were intravenously injected with the priming and eliciting agent at 3-h intervals, and mice were bled 2 h after the second injection as previously reported (22). TNF activities in the sera were measured by a bioassay. As shown in Table III, NOD mice were the lowest producers of endogenous TNF on stimulation with OK-432. When mice were stimulated with other agents, mIFNγ and LPS, much more TNF was induced in the sera compared with stimulation with OK-432 (Table IV). However, NOD mice were also the lowest producers among the various strains of mice. These TNF activities were neutralized with rabbit anti-mouse TNF (data not shown).

Northern blot analysis of TNF mRNA isolated from PEC of various mouse strains. Productivity of endogenous TNF in the different mouse strains was also observed in mRNA levels. RNA was isolated from PEC collected after intraperitoneal stimulation with OK-432 and then subjected to Northern blot analysis. As shown in Fig. 1, bands of TNF-mRNA were detected in the same location near 18S in the different mouse strains. However, the amount of TNF-mRNA was lowest in NOD mice, among the various strains, as was TNF production.

Discussion

OK-432 inhibited insulitis and the development of autoimmune diabetes in NOD mice and BB rats. The precise mechanism of OK-432 action remains unknown, although OK-432 has been shown to inhibit the generation of effector cells for pancreatic B cell destruction in NOD mice (Shintani, S., J. Satoh, et al., manuscript submitted for publication). Because OK-432 induced various cytokines in vivo (18, 19), we screened recombinant lymphokines and monokines for their preventive effects against diabetes using NOD mice. Although only one dose of cytokines was tested and some were of mouse

Table I. Preventive Effect of Various Cytokines on IDDM in NOD Mice

Treatment				No. of mice	Incidence of diabetes	P (χ^2 test)
Cytokine	Dose	Route	Schedule			
	U/mouse				%	
—	—	—	—	118	83.1	
OK-432	0.1 mg	i.p.	1/wk	120	0	<0.001
—	—	—	—	18	77.8	
mIFN γ	1×10^4	i.m.	2/wk	19	68.4	<0.5
—	—	—	—	21	76.2	
hIL 2	8×10^3	i.m.	2/wk	22	59.1	<0.5
—	—	—	—	10	60.0	
mGM-CSF	1×10^4	i.v.	2/wk	10	30.0	<0.25
—	—	—	—	20	70.0	
hIL-1α	6×10^4	i.m.	2/wk	20	45.0	<0.25
—	—	—	—	36	72.2	
hTNFα	3×10^3	i.p.	2/wk	36	22.2	<0.001

Table II. Inhibition of Insulitis in NOD Mice by OK-432 or hTNFα

Treatment	No. of islets counted	Islets (%)			
		Intensity of insulitis*			
		−	+	++	+++
—	62	14.5	22.6	21.0	41.9
OK-432	42	90.5	7.1	2.4	0
hTNFα	81	40.7	22.2	17.3	19.8

* Intensity of insulitis: —, islets without surrounding lymphocytes; +, islets with a few surrounding lymphocytes; ++, islets with many surrounding lymphocytes; +++, islets with intraislets and surrounding lymphocytes.

Table III. OK-432-induced TNF Production in Various Strains of Mice

Mouse strain (n = 4–5)	Priming OK-432 (*20 μg*)	Eliciting OK-432 (*200 μg*)	Serum TNF
			U/ml
NOD	−	+	<0.3
C57BL/6			
C3H/He	−	+	2.2±3.4
Balb/c	−	+	1.1±0.6
NOD	+	+	12.1±10.0
C57BL/6	+	+	22.6±22.9
C3H/He	+	+	106.0±12.6
Balb/c	+	+	621.4±242.3

and others of human origins, the doses of cytokines used in the experiment were of the order of those that showed biological effects in vivo. Among recombinant cytokines, only hTNFα significantly suppressed development of autoimmune diabetes and insulitis in NOD mice.

The suppressive effect of hTNFα on NOD mouse diabetes was significant but not complete, probably because TNF of human origin was used in the experiment. The mitogenic activity of hTNFα on mouse thymocytes was extremely weak compared with that of mTNFα (23), although hTNFα was able to enhance the in vivo immune response in mice (24). Furthermore, NOD mice that were repeatedly injected with hTNFα produced antibodies to hTNFα, which neutralized h-TNFα activity (data not shown). Mouse TNF may have a more potent inhibitory effect on insulitis and development of diabetes in NOD mice.

Recently a direct or indirect role of TNF in the destruction of pancreatic B cells was reported. IFNγ or TNFα enhanced expression of the class I MHC molecule on islet cells (25), which were recognized by $CD8^+$ T cells. In combination, IFNγ and TNFα induced the class II MHC molecule on islet cells in vitro (26) which were recognized by $CD4^+$ cells. Furthermore, TNFα alone or in combination with IL1 or IFNγ was cytotoxic to pancreatic B cells in vitro (27–29). Our result appears

Table IV. mIFN γ- and LPS-induced TNF Production in Various Strains of Mice

Mouse strain (n = 4–5)	Priming mIFN γ (1×10^4 *U*)	Eliciting LPS (*50 μg*)	Serum TNF
			U/ml
NOD	+	−	<0.3
C57BL/6	+	−	<0.3
C3H/He	+	−	<0.3
Balb/c	+	−	<0.3
NOD	−	+	<0.3
C57BL/6	−	+	11.6±4.3
C3H/He	−	+	10.8±3.7
Balb/c	−	+	28.6±14.3
NOD	+	+	1118.3±160.7
C57BL/6	+	+	3332.8±928.9
C3H/He	+	+	1539.6±371.4
Balb/c	+	+	2100.0±713.5

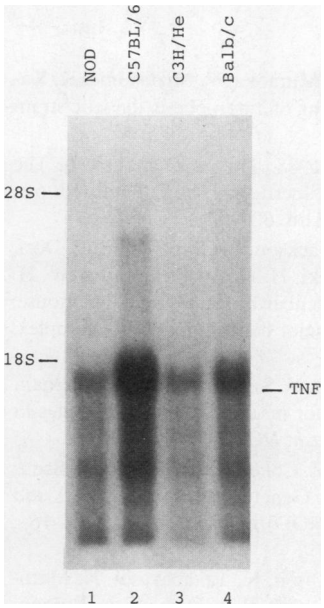

Figure 1. Northern blot analysis of TNF mRNA from the various mouse strains.

to conflict with the observed in vitro effect of TNF on islet cells. However, B cells were exposed to high concentrations of TNF (500–1,000 U/ml) for long periods (2–3 d) in the in vitro experiment, whereas 3,000 U of hTNFα was administered per mouse in our in vivo experiment. It is unlikely that a high concentration of administered TNF was maintained in the blood and microenvironment of the B cells for a long time, because the half-life of TNF administered in vivo is ~ 15 min (30). The administered TNF may have modified the immune systems of the NOD mice rather than directly affected B cell functions.

The mechanisms of TNF action in the inhibition of diabetes is yet unknown. TNF is a protein molecule produced mainly by activated macrophages in response to infection and injury (31). Recently a variety of immunoregulatory functions of TNF has been reported. TNF enhanced natural killer cell activity (32), thymocyte (23), and T cell proliferation (33), expression of IL2 receptors (34), human mixed lymphocyte reaction (35) and immune response to T cell-dependent antigen (24), and suppressed PWM-induced B lymphocyte differentiation (36). It is possible that TNF may modify antiislet autoimmunity in NOD mice and in consequence restore the defective activation of T suppressor cell function (37).

The preventive effect of TNF in the diabetes of NOD mice conversely implies defective immunoregulation by TNF in these mice. Low production of endogenous TNF in vivo on stimulation with OK-432 or with IFN plus LPS was shown in NOD mice. A similar result has been reported in which the administration of exogenous TNF suppressed progression of lupus nephritis in $(NZB \times NZW)F_1$ mice as an animal model of systemic lupus erythematosus (38). The mechanism and role of TNF in suppression of induction and/or progression of autoimmune diabetes need to be further elucidated.

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