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Effects of Complete Freund's Adjuvant(CFA) Treatment on Cytokine Gene Expressions in the Pancreata of Nonobese Diabetic(NOD) Mice

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Type 1 diabetes results from the loss of insulin-producing pancreatic beta cells. T cell-mediated autoimmunity has been suggested to play a crucial role in the destruction of beta cells. Recently local balance between cytokines in the insulitis lesions has been implicated as an important immunologic factor in determining whether diabetes will delvelop or not. In the present study, effects of Complete Freund's adjuvant treatment, which has been known to prevent diabetes in NOD mice, on the expression of cytokine and iNOS gene in the pancreata of NOD female mice were investigated. Histologic examination of pancreatic islets showed that CFA treatment could suppress insulitis. Interestingly CFA treatment was found to stimulate expression of multiple cytokines in the pancreata of NOD mice. Expressions of IL-10, IFN-γ, and iNOS were significantly enhanced, whereas expressions of IL-2, IL-4, and TNF-α were less affected. No changes in IL-12 or TGF-β expression was observed. These results suggest that alterations in cytokine balance, especially an increased expression of IL-10, in the pancreas by CFA treatment may play a role in the prevention of beta-cell destruction.

Key Words: Type 1 diabetes, Immunotherapy, Cytokine, and Complete Freund's adjuvant(CFA)

INTRODUCTION

Type 1 diabetes, which is also called insulin-dependent diabetes mellitus(IDDM), is caused by the absolute deficiency of insulin owing to pancreatic beta-cell destruction. Histologic analysis of the pancreas from patients with recent onset type 1 diabetes revealed an infiltration of the islets of Langerhans by T and B lymphocytes, monocytes/macrophages, and natural killer cells[1,2].

The nonobese diabetic(NOD) mouse, which spontaneously develops type 1 diabetes with quite similar pathological and immunological features, has been used as an excellent animal model for human type 1 diabetes[3]. In NOD mice, insulitis begins to occur around 5~6 weeks of age and apparent insulitis can be observed in all mice of both sexes after 8~9 weeks of age. Differing from humans, NOD mice show a higher prevalence of diabetic syndrome in females(70~80%) as compared to males(10~20%).

Extensive studies have suggested that T cell-mediated autoimmunity play a crucial role in the selective destruction of beta cells[4~8]. Islet-reactive CD4+ and CD8+ T cells have been cloned from insulitis lesions of NOD mice[4,7,8] CD8+ isletspecific cytotoxic T lymphocyte(CTL) may act as a final effector cell, while CD4+ T cells may produce cytokines which in turn activated effectors cells such as CTLs and macrophages. Meanwhile, autoreactive T cells, which were also isolated from insultis lesions, could inhibit beta cell destruction in adpotive transfer models[9]. It is, therefore, believed that the islets of NOD mice may contain both effector cells and cells capable of inhibiting these effector cells, and that the immunoregulatory balance of these cells may be an important factor in determining whether diabetes will develop or not. Although precise mechanisms by which the immune balance is regulated in type 1 diabetes are unkown, a balance between cytokines was suggested to play a crucial role[10]. This hypothesis has been supported by reports that an elevated level of IL-4 and IL-10 expression was associated with protection of grafted islets[11] while an

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increased expression of IFN-γ by infiltrating immunocytes correlated with beta-cell destruction[12].

The spontaneous development of diabetes and autoimmune destruction of grafted islets can be suppressed by *in vivo* treatment with Complete Freund's adjuvant(CFA) and BCG vaccination in animal models[13~15]. Preliminary trial of BCG in newly diagnosed type 1 diabetes patients has been also reported[16]. Although induction of natural suppressor cells and CD4+ immunoregulatory T cells has been suggested to play a role in the prevention of diabetes in CFA-treated NOD mice[14,17], precise mechanisms for the prevention of autoimmune diabetes by these immunostimulatory adjuvants are unknown. In the present study, we report that CFA treatment could alter the cytokine production profile in the pancreata of NOD mice; expressions of IL-10, IFN-γ, and iNOS(inducible nitric oxide synthase) were significantly enhanced, whereas expressions of IL-2, IL-4, and TNF-α were less affected. These results suggest that an alteration in the cytokine profile and enhanced production of nitric oxide(NO) in the pancreas by CFA treatment may play a role in the suppression of autoimmune-mediated beta cell destruction.

MATERIALS AND METHODS

Mice

Our NOD mouse colony was produced from breeding stock originally obtained from Dr. B. Singh at the University of Alberta and was maintained on regular rat chow and tap water ad libitum at the University of Calgary. The cumulative incidence of diabetes at 36 weeks of age in our NOD colony was about 75% for female mice and 15% for male mice.

Treatment with CFA

To see whether CFA injection can affect cytokine expression in the pancreas, 6 week-old female NOD mice were intraperitoneally injected with 50 μl of CFA(GIBCO, Germany). Two weeks and 3 weeks after the injection, pancreatic RNA was prepared for the semi-quantitative RT-PCR analysis.

Histological examination

For histological examination of pancreatic islets, pancreata were fixed with formalin, paraffin-embeded, serially sectioned at 5 μm, and stained with hematoxylin-eosin. Insulitis lesions were graded at follows: 0- normal islet; 1- mononuclear cell infiltration in less than 25% of the islet; 2- 25~50% of the islet infiltrated; 3- over 50% of the islet infiltrated; 4 -small retracted islet with few mononuclear cells.

RNA isolation

Total RNA was isolated from the fresh pancreas by a modification of the acid guanidinium thiocyanate-phenol-chloroform extraction method[18]. Briefly, the pancreas was removed as soon as possible after the animal died and one third of the pancreas(around 60 mg) was immediately homogenized in 4 ml of solution D(4M guanidinium thiocyanate, 17 mM sodium N-lauroylsarcosine, 25 mM sodium citrate, and 0.1M 2-mercaptoethanol) using a homogenizer (Polytron, Brinkman Instruments, Westbury, NY). After one tenth volume of 2M sodium acetate was added, RNA was extracted with water-saturated phenol and chloroform(5 : 1), and precipitated with ethanol overnight. Then, pelleted RNA was rinsed with 75% ethanol, dried, and resuspended in diethylpyrocarbonate(DEPC)-treated distilled water. Concentration of RNA was measured by $O.D._{260}$.

Reverse transcription

Two μg of total RNA was subjected to first-strand cDNA synthesis in a 20 μl of reaction mixture containing 50 mM Tris-HCl(pH8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 μg/ml oligo(dT)12-18, 10 mM dithiothreitol, 0.5 mM of each dNTP, 2 U/μl RNase inhibitor(BRL-GIBCO, Life Technology, Germany), 0.1 mg/ml bovine serum albumin(Pharmacia, Sweden), and 25U Superscript RNaseH- reverse transcriptase(BRL-GIBCO, Life Technology, Germany) for 1 hour at 37℃. After completion of first-strand synthesis, tubes were heated to 95℃ for 5~10 min. The reaction mixture was then diluted to 500 μl with DEPC-treated distilled water and 20 μl was used for each PCR reaction. Reverse transcription reactions were performed simultaneously for samples obtained from various experimental groups and duplicated.

Polymerase chain reaction(PCR)

Twenty μl of cDNA was amplified in a PCR reaction(in a vlume of 50 μl) containing 200 μM of each dNTP, 1 μM of each specific primer(Table 1, University of Calgary DNA synthesis Lab.), buffer supplied by the manufacturer, and 2.5U Taq polymerase(BRL-GIBCO, Life Technology, Germany). PCR was performed in a thermal cycler(Perkin-Elmer

9600, Cetus Corp., Norwalk, CT). In every PCR reaction, cDNA negative control(distilled water) and positive control(Con A-activated spleen cells) were included. To ensure a fixed relationship between the initial input and the densitometric read out, serial dilutions of positive control cDNA (1 : 4 to 1 : 2048, two fold dilution) were amplified at 30 cycles of PCR. NOD pancreas cDNA was amplified at 20, 25, 30, 35, and 40 cycles to choose an appropriate PCR cycle for comparing certain cytokine mRNA expression in the pancreas using semi-quantitative RT-PCR. PCR amplification was performed under following condition; denaturation at 94 ℃ for 1 min; annealing at 60℃ for 1 min; and extension at 72℃ for 1 min. Samples of cDNA to be compared were amplified in the same PCR run and duplicated. PCR product was kept at -20℃ until further analysis.

Southern blot hybridization

Ten μl of the PCR reaction product was electrophoresed through a 1.5% agarose gel in 1X TBE buffer, and then transferred to nylon filter (Zeta Probe; Bio Rad, Richmond, CA). After UV-cross linking, the filters were prehybridized in 6X SSC containing 0.01M NaH_2PO_4, 1mM EDTA, 0.5% SDS, 100 $\mu g/ml$ salmon sperm DNA, and 1% bovine serum albumin at 50℃ for 4 hours. Then the filter was hybridized in a solution identical to prehybridization solution except containing radio-labeled oligoprobe(1 to 2×10^6 cpm/ml of hybridization solution) for 18 hours at 50℃. The filter was washed twice with 6X SSC/0.1% SDS at 52℃ for 30 min, rinsed with 0.1×SSC, and exposed to X-ray film(Hyperfilm-MP; Amersham, Arlington-Height, IL) for the autoradiography. The radioactivity of the bands on autoradiograms was estimated by scanning densitometry (Molecular dynamics). The intensity of bands for cytokine mRNA was divided by the intensity of bands for the internal control, HPRT.

End-labeling and purification of probe using a spun down column

Oligonucleotide probes(Table 1) were end-labeled. 200 ng of a oligonucleotide was incubated in a reaction mixture (20 μl) containing 5 μl of [γ-^{32}P]ATP (6000 Ci/mmol, Amersham), 10 U of polynucleotide kinase (Pharmacia, Sweden), and buffer supplied by the manufacturer at 37℃ for 30 min. Unintegrated [γ-^{32}P]ATP was removed using a spun down column (Centri-Sep column, Princeton Separation, Adelphia, NJ).

Table 1. Oligonucleotide sequences

HPRT	5'	GTAATGATCAGTCAACGGGGGAC
	3'	CCAGCAAGCTTGCAACCTTAACCA
	P	GCTTTCCCTGGTTAAGCAGTACAGCCCC
IL-2	5'	CTTGCCCAAGCAGGCCACAG
	3'	GAGCCTTATGTGTTGTAAGC
	P	GCTTTGAGTCAAATCCAGAACATGCCGCAG
IL-4	5'	TCTTTCTCGAATGTACCAGG
	3'	CATGGTGGCTCAGTACTACG
	P	GACCTCGTTCAAAATGCCGATGATCTCTCT
IL-10	5'	CAAACAAAGGACCAGCTGGAC
	3'	GAGTCCAGCAGACTCAATAC
	P	ATCACTCTTCACCTGCTCCACTGCCTTGCTC
IFN-γ	5'	AGCTCTGAGACAATGAACGC
	3'	GGACAATCTCTTCCCCACCC
	P	GATTTTCATGTCACCATCCTTTTGCCAG
TNF-α	5'	CCTGTAGCCCACGTCGTAGC
	3'	TTGACCTCAGCGCTGAGTTG
	P	CTGGAAGACTCCTCCCAGGTATATGGGTTC
iNOS	5'	CCCTTCCGAAGTTTCTGGCAGCAGC
	3'	GGCTGTCAGAGCCTCGTGGCTTTGG
	P	CAGCTGCTTTTGCAGGATGTCCTGAACGTA
TGF-β	5'	AGGAGACGGAATACAGGGCTTTCG
	3'	ATCCACTTCCAACCCAGGTCCTTC
	P	GCTGAAGCAATAGTTGGTATCCAGGGCTCT
IL-12	5'	ATGGCCATGTGGGAGCTGGAG
	3'	TTTGGTGCTTCACACTTCAGG
	P	TATGACTCCATGTCTCTGGTCTGAGGTCCA

Production of positive control RNA

For a positive control of various cytokine expression, spleen cells from BALB/c mice were stimulated with 5 $\mu g/ml$ of Con A for 20 hours and RNA was isolated.

RESULTS

Histologic examination

To determine whether CFA treatment could prevent insulitis, histologic examination of pancreatic islets from mice aged 9 weeks was performed. As Table 2 shows, significantly lower grade of insulitis was observed in CFA-treated NOD mice as compared to PBS-injected control mice. Although a higher

Table 2. Histologic examination of pancreatic islets

Age[a]	Treatment[b]	Insultis score(%)[c] 0	1	2	3	4	No. of islets examined	Insultis grade (mean±SD)
9 weeks								
	PBS(n=3)	60	23	6	7	4	163	0.7±0.2
	CFA(n=4)	85	12	1	2	0	177	0.2±0.1*

a. Mice were sacrificed at 9 weeks of age.
b. Female NOD mice were injected with CFA or PBS at 6 weeks of age.
c. Percent of islets with the following scores: 0, normal islet; 1, mononuclear cell infiltration in less than 25% of the islet infiltrated; 2, 25 to 50% of the islet infiltrated; 3, over 50% infiltrated; 4, small retracted islet with few mononuclear cells.
*$p<0.05$ as compared with the PBS-injected group, Mann-Whitney test

number of normal islet was observed in CFA-injected mice, CFA treatment could not completely prevent insulitis. Mononuclear cells were found to be localized in the peri-islet lesion and infiltration of mononuclear cells into the islet was observed only in a small number of islet. This result suggested that CFA treatment may inhibit beta cell destruction by suppressing intra-islet infiltration of mononuclear cells.

Semi-quantitative RT-PCR

To ensure successful amplificationh of cytokine mRNA using the specific primers(Table 1), cDNA prepared from Con A-activated spleen cells was amplified for 30 cycles of PCR. Analysis of the RT-PCR product on an agarose gel showed single specific band for the amplification using each set of primers(data not shown).

To ensure a fixed relationship between the initial input and intensity of bands on the autoradiogram, positive control cDNA was serially diluted and amplified using each set of primers. Fig. 1 shows the results the result of autoradiography of RT-PCR products amplified 30 cycles using TNF-α specific primers and hybridized with a specific probe. A similar linear relationship between the concentration of positive control cDNA and the intensity of the band was also observed in the product of RT-PCR using primers and probes for other cytokines.

Expression of cytokine and iNOS gene in the pancreas

To determine whether CFA injection could affect the cytokine

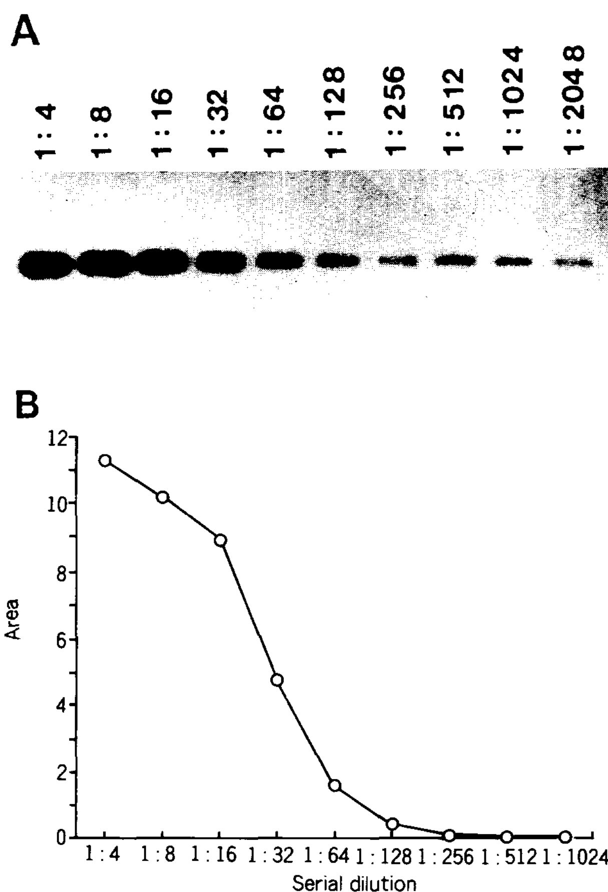

Fig. 1. Relationship between amount of cDNA and densitometric analysis of RT-PCR product. Positive control cDNA(cDNA purified from Con A-activated spleen cells) was serially diluted from 1 : 4 to 1 : 2048 and amplified in 30 cycles of PCR reactions containing primers specific for TNF-α. One fifth of the PCR mixture was electrophoresed through 1.5% agarose gel, and trnasferred to a nylon filter. The filter was hybridized using ^{32}P-radiolabelled TNF-α-specific oligonucleotide probe. Specific bands were detected by autoradiography. The radioactivity of the bands on the autoradiogram(A) was estimated by scanning densitometry(B).

expression profile in the pancreas, female NOD mice were injected with CFA. Cytokine and iNOS expression were analyzed 2 and 3 wk after CFA injection. Significant increases in IFN-γ, IL-10, and iNOS expression were observed in CFA-injected mice at both 2 and 3 weeks after injection(Fig. 2 and 3). A slight increase in IL-2 and IL-4 expression was detected at 2 weeks after the injection and no changes in IL-12 or TGF-β expression was observed(Fig. 3). This result indicated that CFA injection may stimulate expression of IFN-γ, IL-10, and iNOS in the immune cells, whereas IL-2, IL-4, IL-12, TNF-

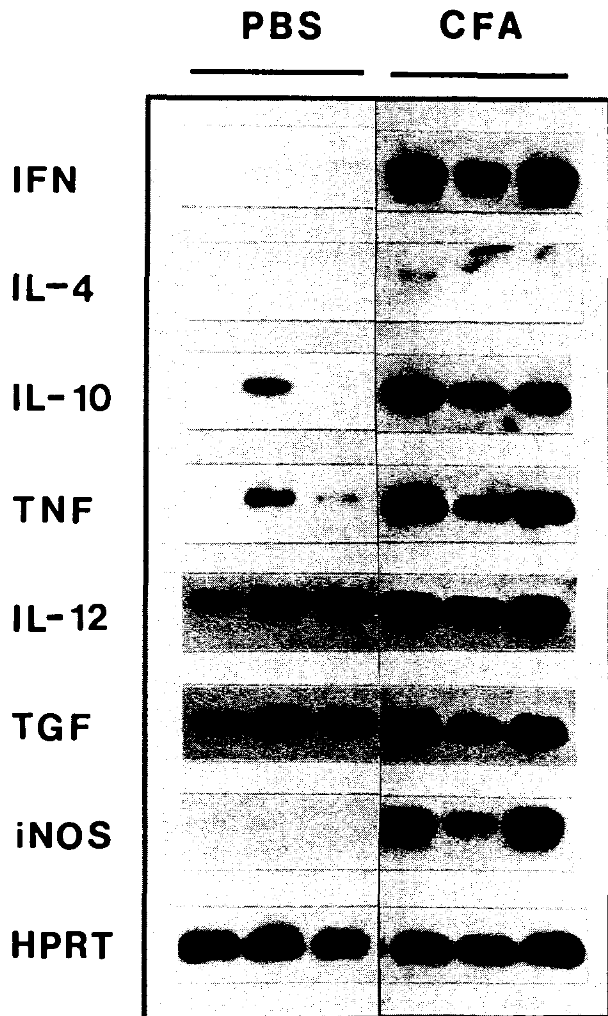

Fig. 2. Effect of CFA treatment on the cytokine and iNOS expression in pancreata of female NOD mice. Mice were injected with CFA at 6 weeks of age and sacrificed 3 weeks after the injection. Two μg of pancreatic total RNA was reverse transcribed and amplified under the conditions specified. One fifth of PCR mixture was electrophoresed through 1.5% agarose gel, and transferred to a nylon filter. The filter was hybridized using ^{32}P-radiolabelled specific oligonucleotide probes. Specific bands were detected by autoradiography.

α, and TGF-β expression were less affected.

DISCUSSION

Treatment with CFA has been reported to prevent development of autoimmune type 1 diabetes in animal models such as NOD mice and BioBreeding(BB) rats[13,14]. In the present study, CFA injection at 6 weeks of age was found to inhibit infiltration of mononuclear cells into the islet and to induce changes in cytokines and iNOS gene expression in the pancreas of NOD female mice. Although precise mechonisms by which CFA injection could suppress the infiltration of mononuclear cells into the islet are unkown, induction of suppressor cells, sch as natural suppressor cells and CD4+ suppressor T cells, has been suggested as possible mechanisms responsible for the prevention of diabetes[14,17]. Since functionally heterogenous T cell populations appeared to be present in the insulitis lesion[4,8,9], presence of mononuclear cells does not always indicate active destruction of the beta-cell. Therefore, infiltrates observed in slets of CFA-injected mice may be somewhat different from those of control mice, either functionally or phenotypically. It can be speculated that less aggressive or protective cells may be dominant in the insulitis lesion of CFA-injected mice.

Expression of a number of cytokines has been reported in insulitis lesions of NOD mice and the balance between the cytokines has been sggested to determine progression of beta cell destruction. CFA injection seemed to stimulate immune cells located in the peri-islet lesions and possibly peri-pancreas lymphoid cells. A remarkable increase in IFN-γ, IL-10, and iNOS expression and a modest increase in IL-2 and IL-4 expression were observed, while IL-12, TNF-α, and TGF-β expression was not significantly affected by CFA injection. The stimulatory effect of CFA injection on expression of these cytokines appeared to persist at least until 3 weeks after injection.

While IFN-γ and nitric oxide have been suggested to cause damage to beta cells[19,20], enhancement of IL-10 expression by CFA injection seems to correlate with the prevention of beta-cell destruction. Administration of IL-10 has been shown to prevent the development of autoimmune type 1 diabetes in mice[21]. IL-10 inhibit cytokine production by murine macrophages and human monocytes, and suppress antigen-stimulated proliferation of and production of cytokines(especially IL-2, IFN-γ, and TNF) by murine Th1 CD4+ T cells[22~24]. Furthermore, IL-10 inhibits TNF-α release and decreases H_2O_2 release by macrophages[25] and inhibit Th1 phenotype development by blocking macrophage production of IL-12[26]. It can be speculated, therefore, that IL-10 locally released in the pancreas may prevent beta-cell destructon in NOD mice by inhibiting functions of effector cells such as CD4+ Th1 cells and macrophages. Acutally, an elevated level

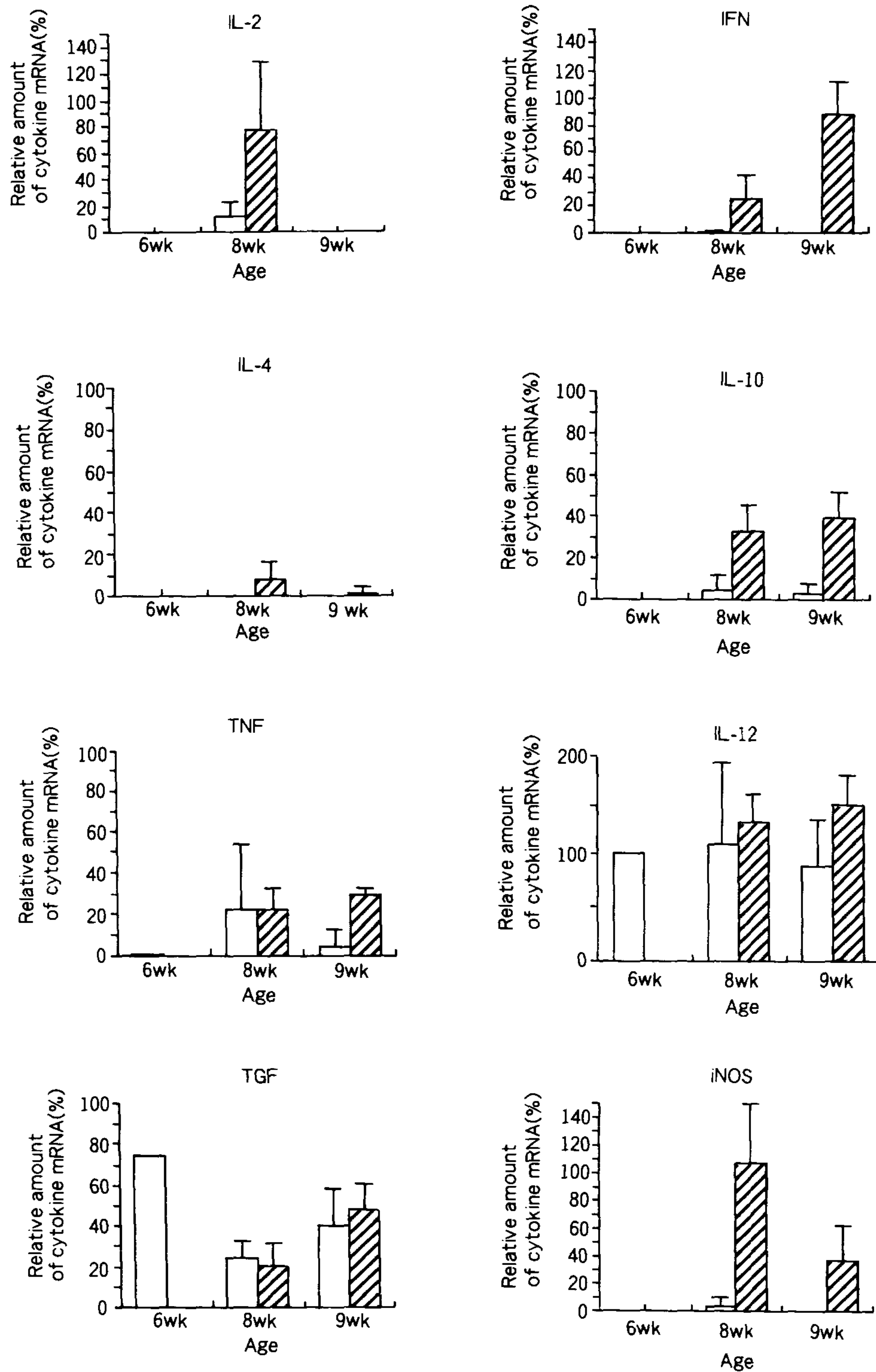

Fig. 3. Effect of CFA treatment on cytokine and iNOS expression in the pancreata of female NOD mice. Bands(IL-2, IFN-γ, IL-4, IL-10, IL-12, TNF-α, iNOS, and TGF-β) on the autoradiograms were analyzed using a densitometer. The amount of cytokine mRNA relative to HPRT mRNA was calculated as follows: cytokine mRNA/HPRT mRNA × 100(%). The error bars indicate the standard deviation of mean relative amount of cytokine mRNA from 3 or 4 mice per group PBS-treated-(□); CFA-treated-(■). *:$p<0.05$ by Mann-Whitney test.

of IL-10 expression correlated with survival of grafted islets in NOD mice[11]. In addition, enhanced expression of IL-10 as well as IL-4 has been associated with the regression of encephalitis in the experimental allergic encephalitis(EAE) model[27]. Whether IL-10 was produced by Th2 cells or by other cells such as CD8+ T cells, B cells, or macrophages in the insulitis lesion of CFA-injected mice remains to be determined.

A role for nitric oxide in the beta-cell destruction has been described. Elevated production of nitric oxide in NOD islets has supported a pathogenic role for nitric oxide[28], and enhanced expression of iNOS as well as IFN-γ was found in NOD mice when development of diabetes was accelerated by cyclophosphamide treatment[29]. Interestingly, however, nitric oxide can inhibit the production of IFN-γ by Th1 cells, while production of cytokines by the Th2 cells appeared to be relatively resistant to inhibitory action of nitric oxide[30]. Recently, Liew et al proposed a hypothesis to explain the effects of nitric oxide on T cells; i.e. nitric oxide at physiologica concentration is required for proliferation of T cells while nitric oxide inhibits cellular proliferation at the high concentrations produced by iNOS[31]. In the present study, CFA treatment was found to stimulate expression of iNOS. If this hypothesis is true, increased production of nitric oxide by iNOS may play a role in the prevention of diabetes in CFA-injected mice by inhibiting proliferation of and cytokine production by Th1 cells. Further studies on the effect of nitric oxide on cellular differentiation and expansion will unravel this possiblity.

CONCLUSION

The present study showed that CFA treatment could change cytokine production profile in the pancreata of NOD female mice. Increased expression of IL-10 appeared to play a dominant role in the prevention of diabetes in CFA-treated NOD mice. Although precise mechanisms whereby IL-10 could inhibit autoimmune-mediated beta-cell destruction remain to be studied, it can be hypothesized that increased expression of IL-10 may inhibit proliferation and activation of effector cells such as Th1 cells and macrophages. In addition, it can be suspected that increased expression of iNOS by CFA treatment may suppress T cell proliferation by inducing production of high concentration of nitric oxide. Results of the present study indicate that the cytokine balance may be an important factor in the pathogenesis of type 1 diabetes and this autoimmune disease may be prevented by a immunotherapy which can change the cytokine balance toward the protective direction.

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