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Interferon-γ plays a role in pancreatic islet-cell destruction of reovirus type 2 -induced diabetes–like syndrome in DBA/1 suckling mice

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Summary. Reovirus type 2 (Reo-2) infection in DBA/1 suckling mice causes insulitis, which leads to pancreatic islet-cell destruction,resulting in a diabetes–like syndrome. T-helper (Th)1 cytokines are thought to play a key role in islet inflammation in insulin-dependent diabetes mellitus. We examined this hypothesis in the Reo-2-induced diabetes–like syndrome. We used reverse transcriptase polymerase chain reaction (PCR) and quantitative PCR techniques to examine mRNA expression of interferon (IFN)-γ (Th1 type cytokine), and interleukin (IL)-4 (Th2 type cytokine) in splenic cells. We observed that in Reo-2 infected mice the level of IFN-γ expression increases with the development of insulitis, whereas expression of message for IL-4 is minimal to detectable with the immuno-inflammatory process 10 days after infection. The treatment of monoclonal antibody (mAb) against mouse IFN-γ during the expansion phase of insulitis (5–9 days after infection) inhibited the development of insulitis and the elevation of blood glucose concentrations in a dose dependent manner. Furthermore altered $CD4^+/CD8^+$ cell ratio compared with uninfected mice in the splenic cells by the infection was recovered to the ratio of uninfected mice by the treatment of mAb against mouse IFN-γ, suggesting normalization of T cell balance in immune system. These results suggest that Reo-2-triggered autoimmune insulitis may be mediated by Th1 lymphocytes and IFN-γ may play a role in islet inflammation leading to islet cell destruction.

Keywords: IDDM, IFN-γ, mouse, reovirus, Th1 cell

Insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease involving an interaction between an epigenetic event, the pancreatic β cells, and the immune system in a genetically susceptible host. Several environmental factors, which trigger the damage of β cells in IDDM, are reported. Possibly viruses, which include coxsackie virus B4 and cytomegalovirus, as well as those causing mumps, varicella zoster and rubella, might be one of the triggering agents of IDDM in man (Notkins 1977; Yoon 1990). Thereafter β cell specific autoimmunity will develop and lead to destruction of these cells (Yoon 1990). In animal models, several

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viruses (e.g. encephalomyocarditis virus, murine cytomegalovirus, coxsackie virus and reoviruses) infect the pancreatic β cell, producing a diabetes–like syndrome (Toniolo & Onodera 1984). Reovirus type 2 (Reo-2) has a striking effect on the pancreatic tissues of DBA/1 suckling mice and produces diabetes–like syndrome. This may represent a suitable animal model (Hayashi *et al.* 1995) for some forms of virus-induced human diabetes (Gepts 1965; Rayfield & Seto 1978). In Reo-2 infection in certain strain of mice a T lymphocyte-mediated autoimmunity is implicated in the pancreatic islet cell destruction correlated positively with hyperglycaemia and hypoinsulinaemia (Onodera *et al.* 1990).

Recent studies have suggested the roles of cytokines in the pathogenesis of IDDM. Antigen-activated T-cells are termed T-helper (Th) cells and at least two distinct Th-cell types, Th1 and Th2, have been reported in mice (Mosmann & Coffman 1989) and in humans (Romagnai 1992). Th1-cells produce interleukin (IL)-2, interferon (IFN)-γ, and tumour necrosis factor-β, whereas Th2-cells produce IL-4, IL-5, and IL-10 (Rabinovitch 1994). Other cytokines are produced by both Th1- and Th2-cell population (Rabinovitch 1994). Th1 cytokines activate cellular immune responses, whereas Th2 cytokines are much more effective stimulators of humoral immune responses (Rabinovitch 1994). Abundant evidence now suggests that Th1 type cytokines especially IFN-γ appear to have diabetes-promoting roles, and Th2 type cytokines especially IL-4 appear to be diabetes-protective, suggesting that IDDM is a Th1-cell-mediated immune process (Debray-Sachs *et al.* 1991; Rabinovitch 1994; von Herrath & Oldstone 1997). However, they are not fully understood to play a role in virus-triggered islet-cell destruction. We examine the role of IFN-γ on the development of insulitis resulting in the destruction of pancreatic islets in Reo-2-triggered diabetes–like syndrome in suckling mice.

Materials and methods

Mice

The animals consisted of 1-day-old DBA/1 J suckling mice of either sex (Kyudo, Saga, Japan). In each experiment, 10 mice were used except for some experiments mentioned below. They were kept in metal cages and fed with commercial pellet (CLEA, CE-2, Tokyo, Japan) and water sterilized by heating to 120°C for 15 min.

Virus

The BN-77 strain of Reo-2 was isolated from a cow with diarrhoea and passaged several times in bovine kidney cells (Kurogi *et al.* 1980) and it was grown on the Hmlu-1 cell line. The Hmlu-1 cells were grown in a growth medium composed of eagle's minimal essential medium (MEM: Nissui, Tokyo, Japan), 10% calf bovine serum (ICN Biomedicals, Osaka, Japan) and 1% tryptose phosphate broth (Difco, Detroit, MI, USA). The culture fluid was centrifuged at 1000 g for 10 min and the resulting supernate was used as virus material after titration. Each mouse was infected with 5×10^5 plaque-forming units/0.05 ml intraperitoneally (i.p.). Infected day was referred as day 0.

Blood glucose tolerance tests(GTTs)

GTTs in control (age-matched uninfected) mice and in mice infected with Reo-2 at 10 days after infection were performed by i.p. injection of 2 mg glucose/gram body weight on nonfasted animals, and glucose concentrations in the serum were determined 60 min later as described previously (Onodera *et al.* 1990).

Treatment of monoclonal antibody (mAb) against IFN-γ

Rat mAb (IgG1: UBI, NY, USA) against IFN-γ, which specifically binds and neutralizes murine IFN-γ and at 1 mg/ml this antibody can detect 10–100 ng murine IFN-γ, was dissolved in phosphate buffered saline (PBS, pH 7.4). Infected mice received i.p. in a dose volume of 0.05 ml in PBS. Mice were treated on five occasions (5,6,7,8 and 9 days after infection) with either mAb against IFN-γ or irrelevant rat immunoglobulin (IgG1, an isotype control for the mAb-treatment: Cedarlane, Ontario, Canada). A total 10 or 50 μg of mAb, respectively, or a total 50 μg of irrelevant immunoglobulin per mouse was administrated. Treatment with mAb started 5 days after infection, since the development of insulitis (expansion phase) begins 7 days after the disappearance of virus from pancreas (Hayashi *et al.* 1995). Suppressive effects by the mAb treatment were evaluated by the GTTs and histology of the pancreatic islets 10 days after infection.

mRNA expression for IFN-γ and IL-4 by splenic cells

A coupled reverse transcriptase polymerase chain reaction (RT-PCR) was used to quantify RNA levels (Wong *et al.* 1994) of splenic cells, which were obtained from either infected mice (3 and 10 days after infection, respectively) or control mice. Each group consisted of four mice. Total RNA was extracted using RNeasy^{TM} Kit

Table 1. Primer sequences for amplification of cytokine and HPRT cDNAs

Gene	Sense or antisense	Sequence	Bases spanned	Amplified band (bp)	No. of cycles
IFN-γ	Sense	5′-TTCTCCTCCTGCGGCCTAGCTCTG-3′	41–64	422	30
	Antisense	5′-GAATGCTTGGCGCTGGACCTGTGGG-3′	438–462		
IL-4	Sense	5′-GACGGCACAGAGCTATTGATGGGTC-3′	70–94	411	31
	Antisense	5′-TAGGCTTTCCAGGAAGTCTTTCAGTGATGTG-3′	450–480		
HPRT	Sense	5′-GTTGGATACAGGCCAGACTTTGTTG-3′	601–625	163	22
	Antisense	5′-GATTCAACTTGCGCTCATCTTAGGC-3′	739–763		

(Qiagen, Hilden, Germany) according to the manufacturer's instructions. Ten μg aliquots of the RNA sample were reverse-transcribed in 25 μl final volume with AMV reverse transcriptase first-strand cDNA synthesis kit (Life sciences Inc., St.Petersburg, USA) according to the manufacturer's instructions. A coupled RT-PCR was used to quantify tissue RNA levels (Wong *et al.* 1994). PCR amplification was performed in a total volume of 25 μl (1.25 U of LA Taq polymerase: Takara Shuzo, Tokyo, Japan), 0.2 μM sense and antisense primers, and 200 μM dATP, dTTP, dGTP, and dCTP, and thereafter overlaid with 20 μl of mineral oil (Sigma, St Louis, MO, USA).

The primers used for amplification are shown in Table 1. Thermal cycling was begun according to the following profile; 1 min at 94°C for denaturation, and 3 min at 68°C for annealing and extension. The optimal number of cycles for PCR amplification was determined experimentally and was defined as the number of cycles that would achieve a detectable concentration well below saturating condition (Table 1: Konecki *et al.* 1982; Gray & Goeddel 1983; Sideras *et al.* 1987). An aliquot of PCR reaction products (approximately 10 μl) was electrophoresed through 2% agarose gels (FMC BioProducts, ME, USA) containing 0.2 μg/ml of ethidium bromide. Gels were illuminated with UV light, and the amounts of amplified products were determined from the densities of the corresponding bands using a Gel Doc 1000 system (Bio-Rad., CA., USA). The results were individually normalized to the signal of the 'house keeping gene', hypoxanthine-guanine phosphoribosyl transferase (HPRT) cDNA, which was amplified in the same tubes for cytokine cDNA amplification. In more detail, PCR was started with primers for a given cytokine species alone, and then the primers for HPRT were added to the reaction tube in an appropriate round of cycling so that the fixed 22 cycles for HPRT and the predetermined optimal cycles (Table 1) for the cytokine could be carried out at the end of the amplification reaction. The specificities of amplified products were confirmed by direct cycle sequencing based on the dideoxy-mediated chain termination method, using a Taq dye deoxy-terminator cycles sequencing kit (Applied Biosystems, CA, USA) after amplified bands were cut out and extracted with glass milk powder (GeneClean II: Bio-101, CA, USA).

Flow cytometry

Flow cytometric analysis for dual colour staining was performed 10 days after infection as described previously (Onodera *et al.* 1991). The spleen was removed aseptically and homogenized and dispersed in MEM as described previously (Hayashi *et al.* 1995). Splenic cells (approximately 1×10^6) from control mice and infected mice with or without mAb treatment (each group consisted of four mice) were suspended in 100 μl of 0.1% sodium azide in PBS (PBS-N) and incubated with 10 μl of a mixture of phycoerythrin-conjugated rat antimouse CD4 (helper/inducer T lymphocyte: CALTAG, CA, USA) and fluorescein isothiocyanate-conjugated rat antimouse CD8 (suppressor/cytotoxic T lymphocytes: Serotec, Oxford, UK) for 1 h and thereafter cells were washed 3 times in PBS-N and then resuspended in 1 ml of PBS-N. Analysis was performed using an EPICS® XL flow cytometer (Coulter, Fla, USA), and fluorescence intensity was expressed by a logarithmic scale. All steps were run at 4°C.

Histopathology

Ten days after infection each pancreas was fixed in 10% neutral buffered formalin (pH 7.4), and sections were stained with haematoxylin and eosin (HE). For each mouse, the incidence (%) of insulitis (immuno-inflammatory cell infiltration into pancreatic islets) including peri-insulitis in the islets was evaluated, and an 'islet score index' was calculated by a method described previously (Hayashi *et al.* 1995). The severity of the cellular infiltration was evaluated by light microscopy, and an insulitis score was given to each islet on a 0–4 scale (0, no peri-insulitis or insulitis; 1, peri-insulitis; 2,

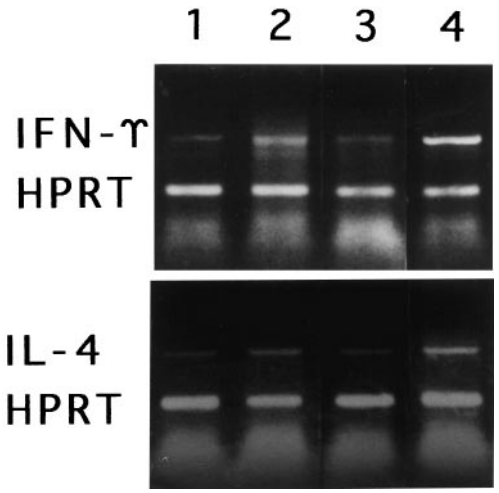

Figure 1. Cytokine gene expression in splenic cells from Reo-2 infected mice (lane 2: 3 days after infection, and lane4: 10 days after infection) and uninfected age-matched control mice (lanes 1 and 3). Expression of IFN-γ and IL-4 messages in splenic cells from groups of four mice was analysed by RT-PCR. The results from one typical sample are shown and only slight variation was detected in other samples.

insulitis < 25% of islet area; 3, insulitis 25–50% of islet area; 4, insulitis > 50% of islet area). The grade of insulitis/mouse was expressed as the average score (score index = total score/number of islets). An average of 20 different pancreatic islets was examined.

Statistical Analysis

The means and standard error (± SE) were calculated and analysed by Student's *t*-test (two-tailed). The accepted level of significance was $P < 0.05$.

Results

mRNA expression for IFN-γ and IL-4 by splenic cells

Three days after infection IFN-γ expression in infected mice (Figure 1, lane 2) was slightly higher than that in uninfected age-matched (control) mice (Figure 1, lane 1). Ten days after infection IFN-γ expression (Figure 1, lane 4) was prominently increased compared to that in control mice (Figure 1, lane 3). There was statistical difference of

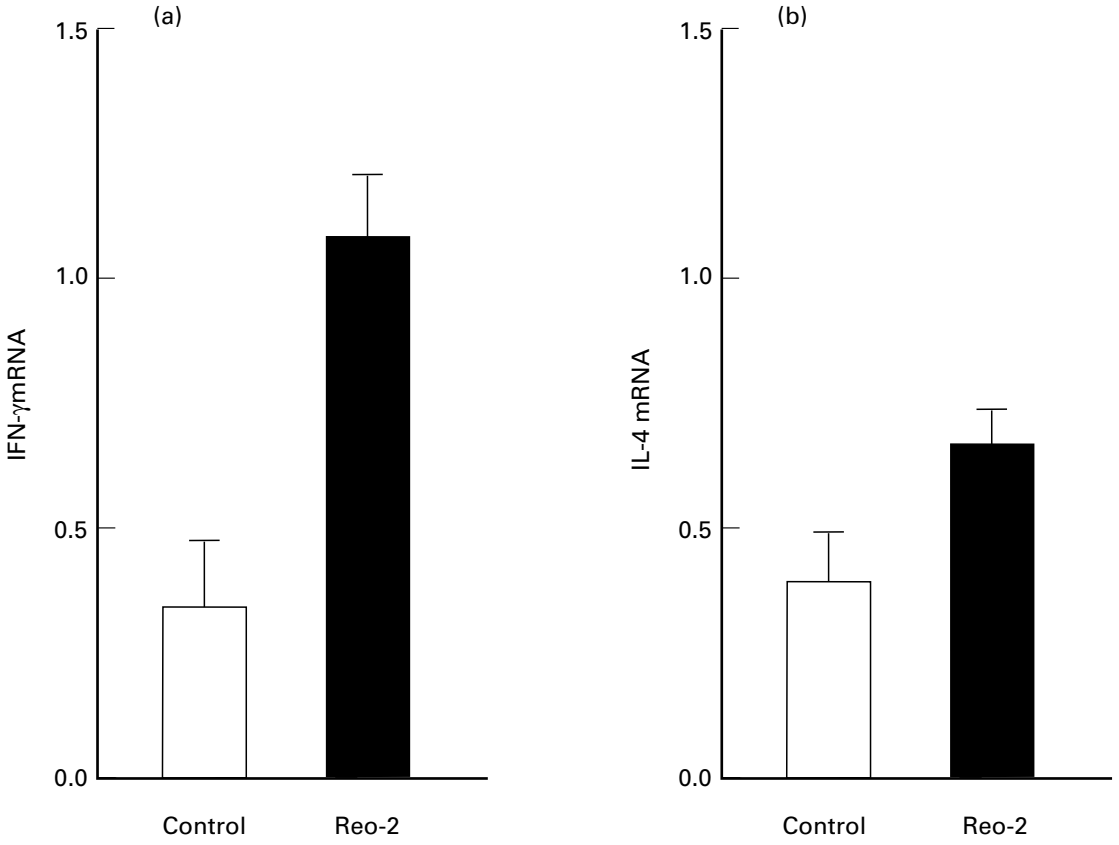

Figure 2. Effect of Reo-2 infection on mRNA expression levels of (a) IFN-γ and (b) IL-4 in spleen cells. All data were individually normalized dividing by the value for HPRT cDNA amplified after the fixed 22 cycles. One-day-old suckling mice were infected with Reo-2 and assays were done 10 days after infection. Data shown are the mean ± SE of four individual mice.

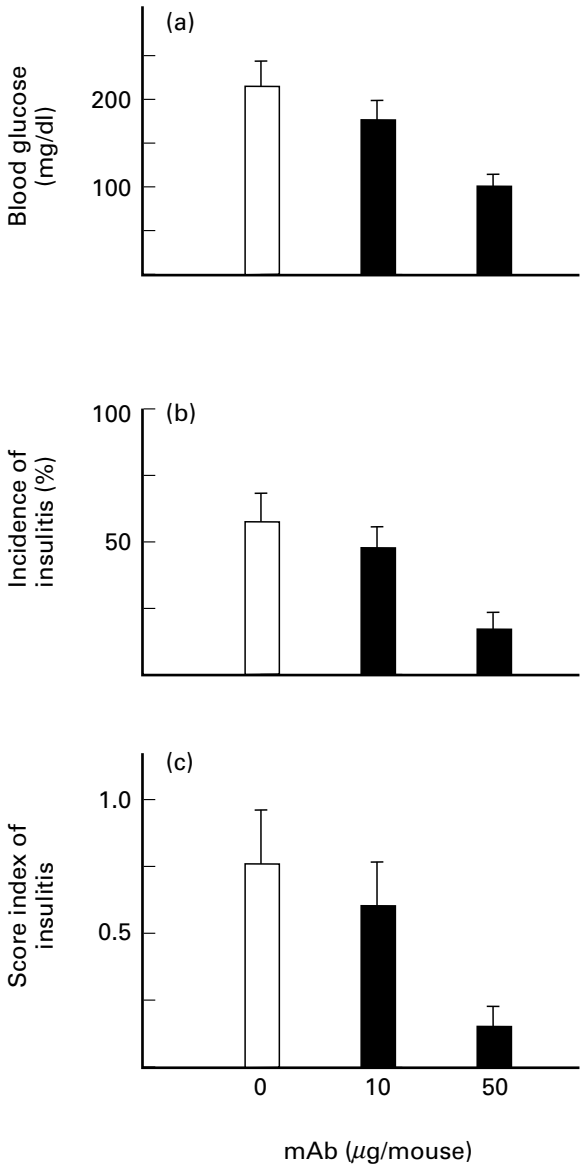

Figure 3. Effect of mAb on (a) glucose elevation, (b) incidence and (c) score index of insulitis in infected mice with (■) or without (□) mAb. The doses treated are shown in the bottom of figure. 0: irrelevant immunoglobulin (50 μg). One-day-old suckling mice were infected with Reo-2 and assays were done 10 days after infection. Each point represents the mean ± SE of 10 mice.

IFN-γ expression between control and infected mice 10 days after infection (Figure 2, $P<0.01$). Whereas IL-4 expression in infected mice 3 (Figure 1, lane 2) and 10 (Figure 1, lane 4) days after infection was slightly increased compared to control mice (Figure 1, lanes 1 and 3, respectively). There was no statistical difference of IL-4 expression between control and infected mice 10 days after infection (Figure 2).

Effect of mAb against IFN-γ on GTTs and insulitis

Ten days after infection there were no differences of blood glucose concentrations (Figure 3a), which were measured 60 min after glucose challenge, and the incidence (Figure 3b) and the score index (Figure 3c) between infected mice treated with irrelevant immunoglobulin and infected mice treated with mAb at a dose of 10 μg. However, those were prominently suppressed by the treatment of mAb at a dose of 50 μg ($P<0.01$). Infected mice showed mean blood glucose concentrations of >200 mg/dl. These differed significantly ($P<0.01$) from the concentrations (about 102 ± 10 mg/dl) in mAb (50 μg)-treated and infected mice. This value was almost the same as control mice (110 ± 5 mg/dl). The incidence of insulitis was 60% in infected mice, whereas in mAb (50 μg) treated group it was 15% ($P<0.01$). The score index in infected mice was 0.70, whereas it was 0.15 in infected mice with mAb treatment ($P<0.01$).

Histopathology

Ten days after infection there was mild to severe cellular infiltration in and/or around pancreatic islets showing cell death (Figure 4a), though those changes varied among islets and mice. Reacting cells consisted of mostly lymphocytes mixed with some neutrophils, eosinophils, macrophages, and plasma cells. Cellular infiltration with oedema and hyperaemia in the interstitial connective tissue, and necrosis of some exocrine pancreatic tissues were also seen. Those changes seen in the infected group were prominently suppressed by mAb treatment at a dose of 50 μg (Figure 4b).

Effect of mAb on lymphocyte subpopulations of splenic cells

The $CD4^+$ (4.8 ± 0.5%)/$CD8^+$ (1.5 ± 0.3) ratio was about 3:1 (Figure 5a) in control mice. In infected mice the $CD4^+$ (8.3 ± 0.5%)/$CD8^+$ (4.6 ± 0.3%) the ratio 10 days after infection was about 2:1 (Figure 5b). Whereas in infected mice treated with mAb at a dose of 50 μg the $CD4^+$ (5.1 ± 0.3%)/$CD8^+$ (1.6 ± 0.3%) the ratio was about 3:1(Figure 5c).

Discussion

The present study demonstrated that the increased expression of mRNA for IFN-γ in splenic cells was paralleled with the development of islet inflammation 10 days after infection. Moreover the treatment of mAb against IFN-γ in the duration of the expansion phase of insulitis (5–9 days after infection) caused suppression of

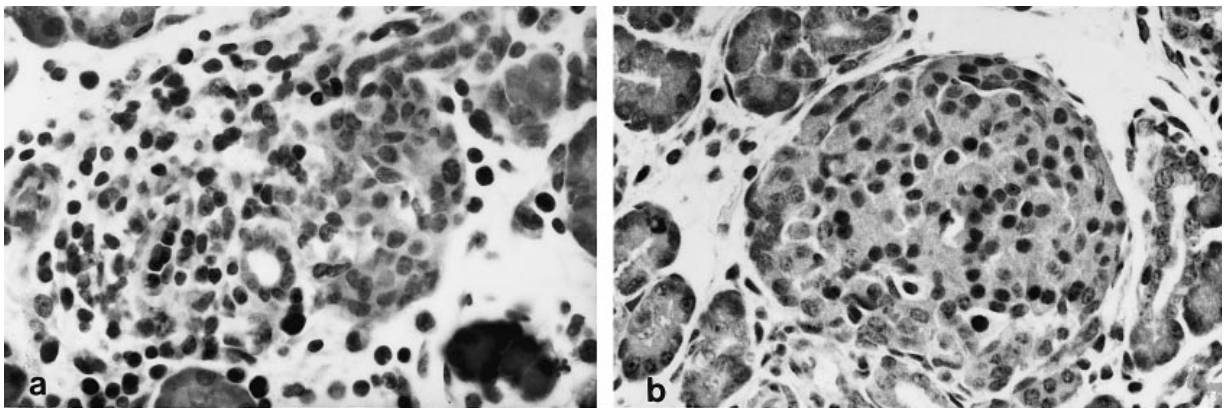

Figure 4. Infiltration of mostly mononuclear cells with polymorphonuclear cells 10 days after infection in and around a pancreatic islet of a mouse infected with Reo-2 (a), whereas cellular infiltration in a Reo-2 infected mouse with mAb (50 μg) is prominently suppressed (b). Representative histological changes are shown in Figure 4. HE. x200.

the development of insulitis and the glucose elevation. Reduction in insulitis following administration of mAb against IFN-γ in nonobese diabetes (NOD) mice, which are an animal model for human IDDM, has been reported (Campbell & Harrison 1989). Furthermore islet-infiltrating mononuclear leucocytes showed high IFN-γ mRNA expression in diabetic BB rats being an animal model for human IDDM (Rabinovitch *et al.* 1996). Taken together the present evidences suggest that IFN-γ may be involved in Reo-2 induced pancreatic islet destruction.

Slight increase in the expression of IFN-γ message 3 days after infection may be due to the viral multiplication, since the viral titres in the pancreas increase 3–5 days after infection (Hayashi *et al.* 1997). It seems likely, since viruses are potent inducers of IL-12 which triggers IFN-γ secretion, the message of IL-12 was transiently increased shortly after infection and this phenomenon is considered to be a common feature of viral infections (Coutelier *et al.* 1995).

IFN-γ has many biological effects both on the islet cells themselves and on the immune system (Kolb & Kröncke 1993). The most striking effects on the immune system and the autoimmune process might be as follows. Firstly, IFN-γ upregulates major histocompatibility complex (MHC) class I expression and induction of the expression of class II glycoprotein on islet β cells and those are a frequent marker during the development of IDDM (von Herrath & Oldstone 1997). Secondly IFN-γ could also contribute to the autoimmune process in the pancreas by bystander activation of autoreactive cytotoxic T (Tc) cells and other immunocompetent cells (e.g. macrophages and natural killer cells) (Nagata *et al.* 1989; Kolb & Kröncke 1993). Thirdly in this virus-induced animal model as we suggested previously IFN-γ may enhance the function and expression of adhesion molecules (intercellular adhesion molecule-1 and lymphocyte function-associated antigen-1), which participate in the many stages of an autoimmune responses (Campbell *et al.* 1989; Springer 1990; Hayashi *et al.* 1995).

As evidenced here, the expression of IL-4 mRNA is minimal as compared to IFN-γ mRNA expression 10 days after infection. This may be resulted in the inhibition of the production of IL-4 (Th2 cytokine) by IFN-γ (Th1 cytokine), since responses of Th1- and Th2-cells are mutually inhibitory (Rabinovitch 1994). Thus Th1-cell rather than Th2 cell may play a role in the development of Reo-2-induced insulitis. This idea is strengthened by the reports that IL-4 production is decreased in NOD mice (Rapoport *et al.* 1993) and that administration of IL-4 can prevent insulitis and diabetes development (Rapoport *et al.* 1993). Moreover IL-12, which promotes the development of Th1 cell, accelerated autoimmune diabetes in NOD mice (Trembleau *et al.* 1995).

Taken together IFN-γ may effect on immunocompetent cells outside of the pancreas (lymphoreticular tissues such as spleen and lymphnodes, etc.), and may operate at a site of inflamed pancreatic islets (Rapoport *et al.* 1993; Fox & Danska 1997) as discussed above.

Infected mice showed increased $CD8^+$ population resulting in decreased $CD4^+/CD8^+$ cell ratio compared to control mice, suggesting that $CD8^+$ T cell, which has been shown in the role of destructive phase of autoimmune diabetes (Kolb & Kröncke 1993), may generate in the presence of IFN-γ. In addition this decreased $CD4^+/CD8^+$ cell ratio by infection was recovered to control levels by the treatment of mAb, suggesting that the imbalance of T cell subpopulation in infected mice may be normalized by the treatment of mAb, resulting in the suppression of the development of insulitis. This seems likely, since we have previously reported that

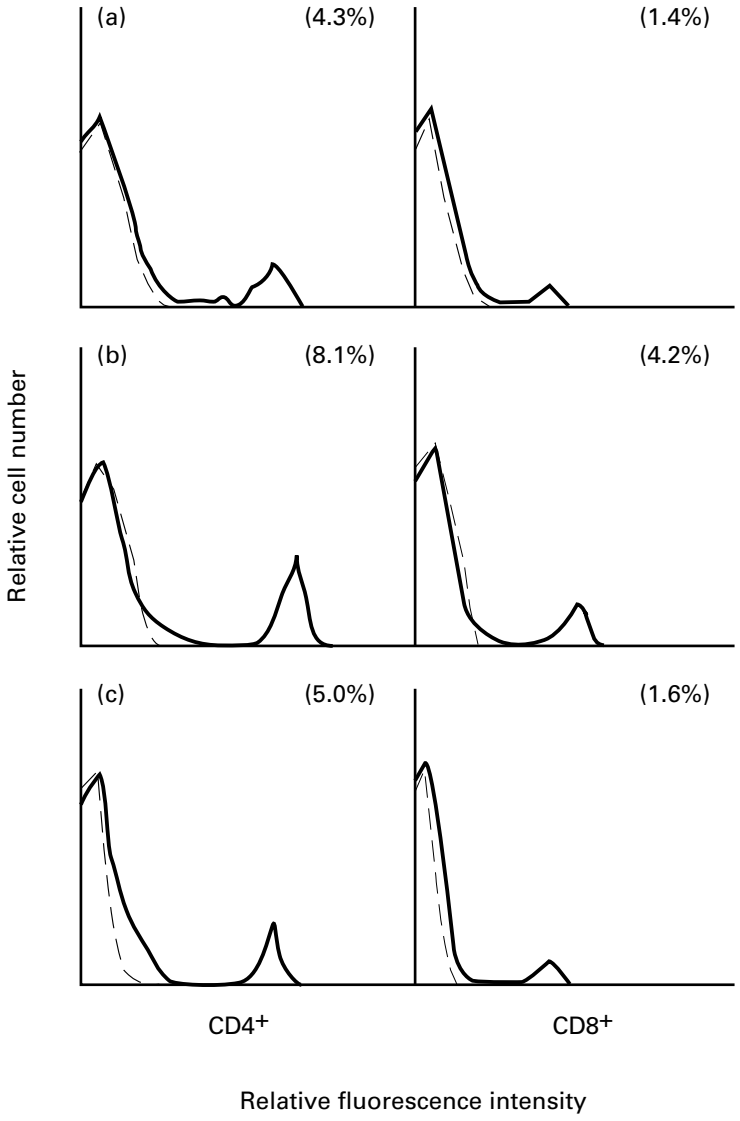

Figure 5. Flow cytometric analysis of $CD4^+$ and $CD8^+$ expression on splenic cells in (a) a control mouse, (b) a Reo-2 infected mouse and (c) a Reo-2 infected mouse treated with mAb (50 μg). —— Profiles of stained cells; - - - - Background staining (cell autofluorescence). The numbers express percentage of positively stained cells. One-day-old suckling mice were infected with Reo-2 and assays were done 10 days after infection. The results shown are the representative of four separate experiments.

abnormal immune responses in Reo-2-infected mice were recovered by the treatment of immunomodulator such as thymic hormone, resulting in increased $CD4^+$/$CD8^+$ cell ratio of the thymus (Onodera *et al.* 1991).

Reo-2 may destroy part of the pancreatic islet cells by direct infection without inducing abnormal glucose tolerance (Onodera *et al.* 1990; Hayashi *et al.* 1995). Then the host antigens released from the damaged β cells being immunogenic (Wilson & Leither 1990) are processed by antigen presenting cells (e.g. macrophages and dendritic cells) and thereafter presented to Th cells in association with MHC class II molecules in lymphoreticular organs and in the pancreas (Yoon 1990; Nossal *et al.* 1992). Subsequently secreted Th1 type cytokines such as IFN-γ activate Tc cells including macrophages and natural killer cells (Rabinovitch 1994). At the same time reovirus-induced increased expression of MHC class I molecules coupled with the host antigens on murine pancreatic islet β cells (Campbell *et al.* 1988) may be recognized by Tc cells. This will lead to islet cell destruction causing abnormal glucose tolerance. Further studies are necessary to explain the Th1/Th2 balance (Brennan & Feldman 1992; Fitch *et al.* 1993; Lenschow *et al.* 1996) in the development of Reo-2-induced autoimmune diabetes, since it has been reported that Th2 cells also could be involved in the disease process (Tian *et al.* 1997). This is the first evidence for participation of IFN-γ in Reo-2 induced islet cell destruction. These findings may help to elucidate some forms of human virus-induced IDDM.

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