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Viruses and Cytokines: Evidence for Multiple Roles in Pancreatic Beta Cell Destruction in Type 1 Insulin-Dependent Diabetes Mellitus

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Insulin-dependent (type 1) diabetes mellitus (IDDM) is due to the selective autoimmune-mediated destruction of pancreatic beta cells possibly initiated by viruses. To elucidate the possible role of viruses and cytokines in the pathogenesis of IDDM, we have examined the effect of reovirus infection on beta cell major histocompatibility complex (MHC) expression and the effect of interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) on beta cell function in vitro. Infection of RIN-m5F (rat insulinoma) cells with reovirus-1 or reovirus-3 was associated with a tenfold increase in class 1 MHC protein and mRNA expression. Reovirus infection did not induce the expression of class 11 MHC by RIN-m5F cells. Exposure of reovirus to ultraviolet light almost completely abolished its ability to induce class 1 MHC protein expression on infected cells.

Murine islets cultured for 3 days with IFN-γ and/or TNF-α had a significantly reduced insulin response to glucose, which was more marked with a combination of the cytokines. During 6 days of culture in IFN-γ plus TNF-α islets underwent noticeable degeneration associated with an 80% reduction in insulin content. These findings together with previous data suggest viruses and cytokines may have multiple roles in beta cell destruction, indirectly through enhanced MHC protein expression and directly through functional impairment and loss of viability.

Key words: IFN- and TNF-pancreatic beta cell function, insulin secretion and content, effects of IFN- and TNF-reovirus infection, major histocompatibility complex protein expression, RIN-m5F cells major histocompatibility complex protein expression, major histocompatibility complex, mRNA levels

The majority of cases of type 1 or insulin-dependent diabetes mellitus (IDDM) appears to be the result of an autoimmune disease in which the insulin-producing pancreatic beta cells are selectively destroyed. The exact pathogenic events and mechanism(s) of beta cell destruction in IDDM are unknown. It is currently speculated that in genetically susceptible individuals, viral infection or chemical damage to the beta

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cells triggers an immune reaction which progressively destroys the beta cells and which is evidenced by both humoral and cellular immune markers [1,2].

A dominant pathologic finding in persons with newly diagnosed IDDM is the infiltration of pancreatic islets with mononuclear cells, the majority of which are T lymphocytes [3–5]. This so-called insulitis, found in close association with insulin-containing islets [5], suggests a crucial role for cellular autoimmunity in mediating beta cell destruction in IDDM. A further feature in the islets is the hyperexpression of class 1 and the aberrant expression of class 11 major histocompatibility complex (MHC) proteins [6]. These alterations in MHC protein expression apparently can be seen in the absence of immunoinflammatory cells suggesting they may precede insulitis.

Some viruses have been implicated in the pathogenesis of IDDM [7]. We hypothesize that candidate viruses, upon infection of pancreatic beta cells, upregulate MHC protein expression. Such an effect would precede and promote the onset of insulitis. With insulitis, cytokines such as interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) produced by infiltrating T cells and monocytes would further modify MHC protein expression and possibly beta cell function and viability.

The aim of the present experiments was to examine the effects of reovirus infection on beta cell MHC protein expression and the effects of IFN-γ and TNF-α on beta cell function and viability. Reovirus is an appropriate candidate virus, as it has been shown to infect beta cells [8,9] and to induce polyglandular autoimmunity, including diabetes, in mice [8,10].

MATERIALS AND METHODS

Cells and Islet Isolation

A rat beta cell line (clone RIN-m5F; kindly supplied by Dr H. Oie, National Cancer Institute, Bethesda, MD) was grown continuously in monolayer culture in RPMI-1640 plus 5% FCS. Isolated islets were prepared by the collagenase digestion technique from the pancreata of overnight fasted CBA mice. The islets were cultured free floating in bacteriological grade petri dishes containing RPMI-1640 plus 5% FCS.

Reovirus Infection of RIN-m5F Cells

Cultures of RIN-m5F cells were infected with either reovirus-1 (RCH-1144 strain) or reovirus-3 (Dearing strain) as described previously [11].

Cytokine Treatment of Islets

Groups of 50 islets were cultured in the presence or absence of IFN-γ and/or TNF-α in 7 ml of RPMI-1640 plus 5% FCS. Murine recombinant IFN-γ (specific activity 2.2×10^7 U/mg) and TNF-α (specific activity 2.8×10^7 U/mg) were generously supplied by Dr H. Michael Shepard, Genentech Inc. (San Francisco, CA).

Analysis of MHC Protein Expression

MHC protein expression was analysed 48 h after reovirus infection of RIN-m5F cells. Expression was quantitated by flow cytofluorimetric analysis of approximately 10,000 stained cells using an EPICS model 751 cell sorter (Coulter Corp., Hialeah, FL). Staining of cells for MHC proteins was performed as described previously [12] using monoclonal antibodies to class 1 (34-1-2S) and class 11 (OX-6; purchased from

Serotec, Oxford, UK). A monoclonal antibody reacting with the murine H-2K^b protein was used as a negative control. Briefly, cell suspensions were prepared by trypsin (0.2% w/v) treatment of the monolayer cultures. The cells were resuspended in 10 ml of growth medium and incubated at 37°C for 60 min prior to staining. Aliquots of the cell suspensions (1 ml containing $\sim 5 \times 10^5$ cells) were centrifuged in 10 ml plastic tubes. Fifty microlitres of the appropriate monoclonal antibody were added to the cell pellets, which were resuspended and incubated at 4°C for 30 min. After washing twice in PBS containing 1% FCS, 50 μl of fluorescein-conjugated rabbit antimouse immunoglobulin (Silenus, Melbourne, Australia) diluted 1:30, was added and the cell pellets were resuspended and incubated at 4°C for 30 min. Cells were finally washed twice before resuspension in 1 ml of 2% (v/v) formalin in PBS containing 1 mg/ml bovine serum albumin.

Northern Blot Analysis of Poly(A+) RNA

Following 24 h reovirus infection, approximately 10^8 cells were resuspended in 6 ml STE buffer (0.1 M NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA) containing 60 μl 20 mg/ml proteinase K and 300 μl 10% sodium dodecyl sulfate (SDS). The mixture was homogenized for 30 s at high speed in a Polytron and then incubated for 60 min at 37°C. After the addition of 60 μl 5 M NaCl, poly(A+) RNA in the digest was extracted on to oligo (dT) cellulose (1 ml 40 mg/ml) in loading buffer consisting of 0.5 M NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 0.1% SDS. After mixing by rotation for 1–2 h at room temperature the oligo (dT) cellulose was centrifuged and washed with 3 × 6 ml loading buffer. Bound poly(A+) RNA was then eluted in the same buffer minus NaCl, precipitated in ethanol and stored at −20°C.

The concentration of RNA was determined by absorbance at 260/280 nm. Five micrograms of RNA denatured by heating for 5 min at 65°C in MOPS electrophoresis buffer (20 mM morpholinopropanesulfonic acid, pH 7.0; 5 mM sodium acetate; 1.0 mM EDTA) was loaded per lane on a 1% agarose gel containing 6% formaldehyde. After electrophoresis at 60 V for 2–3 h, RNA in the gel was stained with ethidium bromide and, after being photographed, was transferred overnight by capillary action onto nylon membrane (Zeta probe Bio-Rad, Richmond, CA) under alkaline conditions (5 mM NaOH, 1.5 M NaCl). The membrane was washed in 2 × SSC buffer (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0).

The probes used were a 1,400 base pair (bp) *Pst1* fragment of a cDNA clone for human HLA-B [13] shown by us to cross-hybridize with rat class I MHC sequences [14]. The probe was labeled with [α-^{32}P] dATP to a specific activity greater than 10^8 CPM/μg, using the random oligonucleotide primer method [15].

Prehybridization for 2 h at 42°C and hybridization overnight at 42°C were performed in buffer consisting of 2 × SSPE (1 × SSPE is 0.18 M NaCl; 10 mM NaH_2PO4, pH 7.4; 1 mM EDTA), 5% formamide, 0.5% skim milk powder, 7% SDS, and 500 μg/ml salmon testis DNA.

The membrane was then washed in 3 × SSC buffer prior to autoradiography.

Functional Evaluation of Islets

Islets treated with or without cytokines were preincubated for 90 min in Hepes-buffered Krebs-Ringer bicarbonate buffer, pH 7.6, containing 5 mM $CaCl_2$/mg/ml BSA and 2 mM-D-glucose (HKRB buffer). Groups of five islets were then placed in 1 ml of the HKRB buffer with either 2 mM or 20 mM glucose and incubated for a

further 60 min at 37°C. Following centrifugation, supernatants were removed and stored at −20°C pending assay for insulin by radioimmunoassay, using rat insulin as standard.

For the determination of insulin content, groups of 20 islets previously treated with or without cytokines were collected in polypropylene microfuge tubes, washed twice in PBS containing 1% FCS, and extracted in 0.5 ml of acid-ethanol solution (1 M HCl:H_2O:95% CH_3CH_2OH:200:10:790 v/v). Extraction was completed by ultrasonication of the islet solutions for 10 s. Acid ethanol extracts containing solubilized insulin were diluted in PBS containing 1% FCS prior to insulin radioimmunoassay.

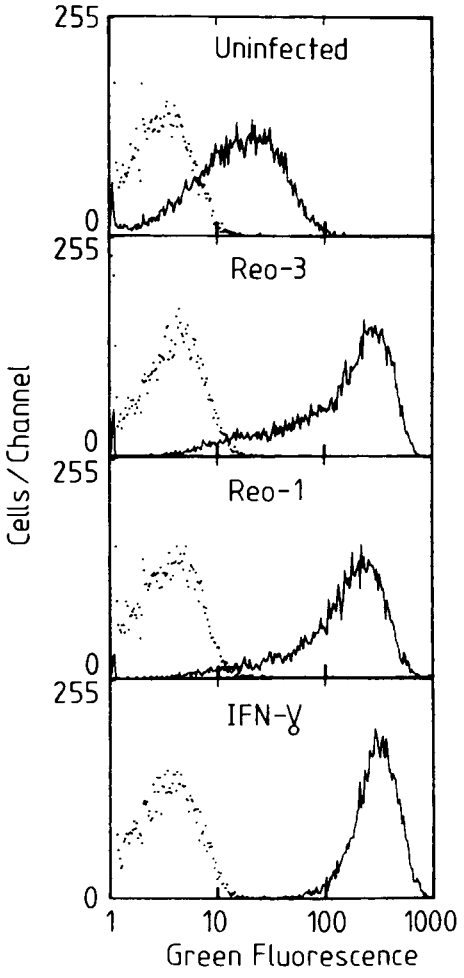

Fig. 1. Class 1 MHC protein expression by RIN-m5F cells infected with reovirus or treated with IFN-γ (100 U/ml). Following infection or treatment with IFN-γ, cells were incubated with monoclonal antibody 34-1-2S to rat class 1 MHC proteins and stained with fluorecein anti-mouse Ig. Approximately 10,000 stained cells were analysed by flow cytofluorimetry (solid line). Dotted line represents the non-specific background staining obtained with control monoclonal antibody.

RESULTS

Reovirus and MHC Protein and mRNA Expression by RIN-m5F Cells

At 48 h, following infection with either reovirus 1 or reovirus 3, staining for class 1 MHC proteins was increased by up to tenfold above levels on uninfected cells (Fig. 1). In comparison, as shown previously [14] class 1 MHC protein expression on cells treated with IFN-γ (100 U/ml), was increased up to 15-fold (Fig. 1).

These effects of reovirus infection and IFN-γ were mirrored by changes in the level of class I MHC mRNA detected by Northern blot analysis (Fig. 2). The cDNA probe hybridized to RIN-m5F cell RNA migrating at 2.0 kilobases.

RIN-m5F cells do not express class 11 MHC proteins; following infection of RIN-m5F cells with reovirus-3 there was no induction of the class 11 MHC proteins (Fig. 3).

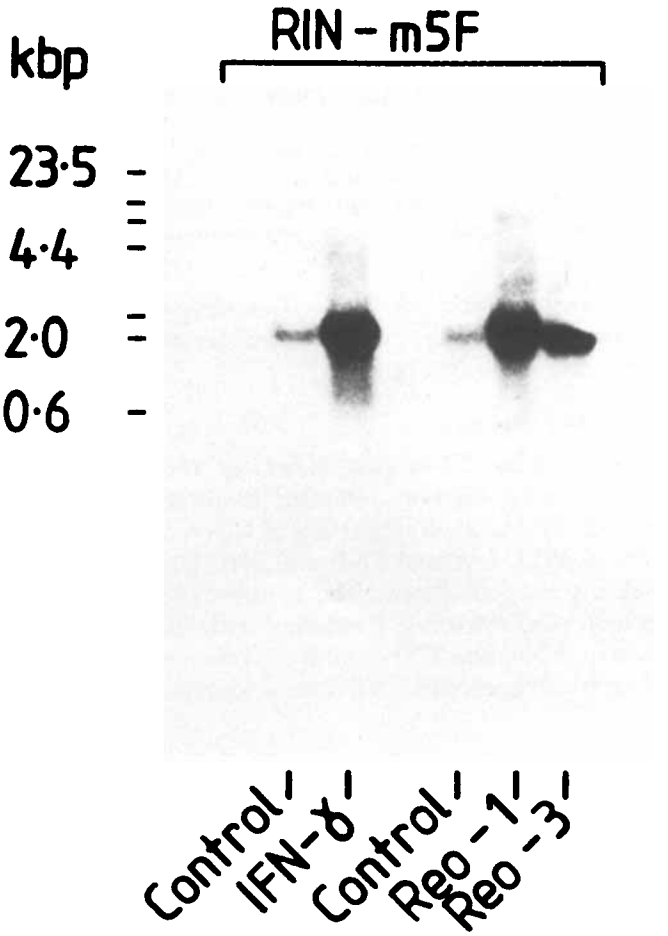

Fig. 2. Class I MHC mRNA expression in RIN-m5F cells. Northern blot hybridization of poly(A+) RNA from RIN-m5F cells infected with or without reovirus or exposed to IFN-γ (100 U/ml) for 24 h.

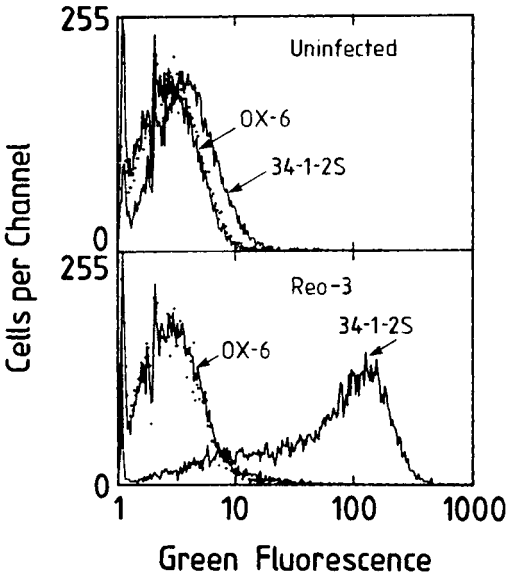

Fig. 3. Effect of reovirus-3 infection on class 1 and class 11 MHC protein expression by RIN-m5F cells. Some 48 h following infection, cells were stained for class 1 (34-1-2S) or class 11 (OX-6) protein expression. Approximately 10,000 stained cells were analyzed by flow cytofluorimetry (solid lines). Dotted line represents the nonspecific background staining obtained with control monoclonal antibody.

Exposure of reovirus-1 or reovirus-3 to ultraviolet light resulted in a sixfold reduction in the staining for the class 1 MHC proteins on infected cells compared with cells infected with native reovirus (Fig. 4).

Cytokines and Islet Function

Islets exposed to either IFN-γ (500 U/ml) or TNF-α (500 U/ml) for 48 h showed a marked decrease in glucose-stimulated insulin release (Table I), which was accentuated after exposure to the combination of IFN-γ and TNF-α. After a 6 day exposure to IFN-γ (2,000 U/ml) and TNF-α (2,000 U/ml) morphological integrity of the islets was clearly lost (not shown). This morphological effect was not seen with islets exposed to individual cytokines. Consistent with these morphological appearances islets exposed to IFN-γ and TNF-α for 6 days contained only 20% of the insulin in nonexposed islets; islets exposed to TNF-α alone also showed a reduced insulin content (Table I).

DISCUSSION

Our findings suggest that viruses and cytokines may have multiple pathogenic effects in the pathway of beta cell destruction leading to the development of IDDM. These effects appear to be operative at at least two levels, viz., the up-regulation of beta cell surface MHC protein expression and the inhibition of beta cell function with associated loss of viability.

While we have not formerly reported cytopathic effects of reovirus on the beta cell in the present paper, these have been observed by us and described by other work-

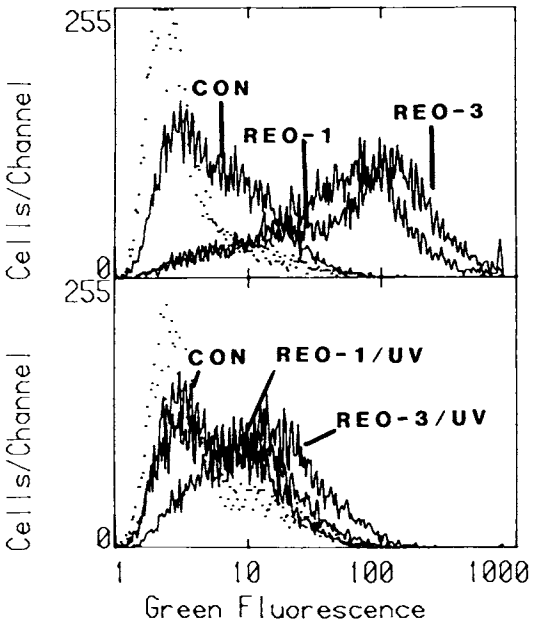

Fig. 4. Effect of UV irradiation on reovirus-enhanced class 1 MHC protein expression by RIN-m5F cells. Some 48 h following infection with native (**top**) or UV-irradiated reovirus (**bottom**), cells were stained for class 1 MHC protein expression and analyzed as described for Figure 1.

ers [9]. It is clear that in addition to these cytopathic effects reovirus infection of the beta cell is accompanied by a significant up-regulation of the class 1 MHC proteins. Such an effect of reovirus is not limited to the tumoral RIN-m5F cell but has also been recently shown by us to occur in normal cultured human beta cells following infection [11]. The present studies confirm and extend these previous findings and show that reovirus infection is almost as potent as IFN-γ in the up-regulation of not only class 1 MHC proteins but also class I MHC mRNA levels in RIN-m5F cells. Although the mechanism for this effect is not known, it is tempting to speculate that reovirus may

TABLE I. Effects of IFN-γ and TNF-α on Glucose-Stimulated Insulin Release From, and Insulin Content of, Mouse Islets†

Treatment	Insulin released (ng/islet/60 min)[a]		Insulin content (ng/islet)
	2 mM glucose	20 mM glucose	
Nil	0.8 ± 0.1	13.6 ± 1.4	38.2 ± 2.4
IFN-γ	0.7 ± 0.05	7.3 ± 1.6*	33.1 ± 2.6
TNF-α	0.9 ± 0.04	6.4 ± 1.3*	21.5 ± 4.7*
IFN-γ + TNF-α	0.6 ± 0.1	2.8 ± 0.7*	6.6 ± 2.6*

[a]Results are expressed as mean ± SEM for five observations.
†Islets were cultured for 48 h with or without IFN-γ (500 U/ml) or TNF-α (500 U/ml) prior to glucose challenge. Islets were cultured for 6 days with or without IFN-γ (2000 U/ml) or TNF-α (2000 U/ml) prior to extraction of insulin.
*For significance, $P < 0.05$ (Student's t-test) compared with nontreated islets.

directly increase class I MHC mRNA transcription and/or stability. In support of this there is a loss of effect after UV treatment of reovirus, suggesting a requirement for productive virus infection rather than just the presence of double-stranded RNA. Double-stranded RNA has been shown to be more potent than native reovirus in inducing interferon production from cells [16]. In addition we previously found that virus-depleted supernatants from infected RIN-m5F cells do not stimulate class 1 MHC protein expression [11]. It is therefore unlikely that reovirus-infected RIN-m5F cells secrete factors such as IFN-α/β, which then feed back to stimulate class 1 MHC protein expression. We have also found that rat fibroblast IFN preparations only weakly enhance class 1 MHC protein expression compared with reovirus infection or IFN-γ (unpublished data). The possibility that reovirus infection induces a second messenger such as an IFN that is active entirely intracellularly is not excluded and is the subject of active investigation in our laboratory.

The question arises as to the significance of reovirus-induced up-regulation of class 1 MHC proteins on the beta cell. Clearly this effect is independent of cytokines produced by immunoinflammatory cells such as IFN-γ and TNF-α, which we have also shown to up-regulate beta cell class 1 MHC proteins [12,14,17]. In relation to beta cell destruction in IDDM, up-regulation of class 1 MHC proteins by reovirus infection might lead to increased targeting and cytolysis by class 1 restricted cytotoxic T cells. Such a concept is supported by experimental studies showing that virally infected cells with up-regulated class 1 MHC proteins are much better targets and are killed more efficiently by cytotoxic T cells [18]. It is of great interest that Foulis and colleagues [19] reported that residual beta cells in 33 of 34 pancreata obtained at autopsy from newly diagnosed diabetes contained immunoreactive IFN-α in association with the hyperexpression of class 1 MHC proteins. Our findings provide support for the role of viruses in initiating the histopathological changes noted by Foulis and colleagues. Enhanced class 1 MHC protein expression by the pancreatic beta cell may represent a direct cellular response to virus infection, independent of the action of cytokines such as IFN-γ and TNF-α which are released by immuno-inflammatory cells. Hyperexpression of class 1 MHC proteins on beta cells in response to virus infection could initiate the development of insulitis either by targeting T cells to viral antigen or by directly impairing beta cell viability [9] with the induction of an immune response to altered/shed beta cell antigens.

The present studies show that, in addition to their previously described ability to induce class 1 and class 11 MHC proteins on islet cells [14,20], IFN-γ and TNF-α inhibit directly the function and viability of murine islets. The cytokines alone caused a marked decrease in glucose-stimulated insulin release which was further accentuated when the cytokines were used in combination. Synergism between the cytokines to mediate cytotoxicity to the islets was evidenced by loss of morphological integrity and significantly reduced insulin content. These findings are consistent with the recent report by Pukel et al. [21], who observed synergistic cytotoxicity of IFN-γ (10^2 U/ml) and TNF-α (10^3 U/ml) over 4 days to monolayer cultured rat islet cells using a ^{51}Cr release assay. The dose and duration of treatment required for rat islet cell killing in the experiments of Pukel et al. were therefore similar to those we have used in the present report. Our findings suggest that cytokines produced by activated T lymphocytes and macrophages within the insulitis lesion directly block function and eventually kill the beta cell. It is not clear whether these toxic effects are mediated in vivo by secreted cytokines acting in a microenvironment or by cytokines delivered directly into

the target beta cell by contact with the T lymphocyte or macrophage, as postulated for T-helper lymphocyte activation of the B lymphocyte [22].

Numerous cytokines, including IFN-γ and TNF-α, play a pivotal role in regulating various pathways of the immune response [for a review see 23]. However, it is now clear that in addition to their immunoregulatory functions, many cytokines have wide ranging hormonelike effects, influencing the growth and function of most cells [23–25]. Many of the cytokines have synergistic effects, e.g., IFN-γ and TNF-α are antiviral [26] and cytotoxic [27] to various cells, in combination but not alone. The studies herein expand the list of pleiotropic actions of IFN-γ and/or TNF-α. They also add IFN-γ and TNF-α to IL-1 [28] as cytokines that directly modulate islet cell function and viability.

Support for the role of cytokines in the molecular pathology of autoimmune disease has recently been provided by Powell and colleagues [29] who found that the ability of T cell lines raised against myelin basic protein to transfer experimental allergic encephalomyelitis in rats was related to their production in vitro of lymphotoxin. Finally, in vivo studies demonstrate that IFN-γ enhances the severity of the autoimmune lupus syndrome in NZ B/W mice [30] and autoimmune diabetes in mice following multiple low-dose streptozotocin [31].

In conclusion, our findings suggest that viruses and cytokines have multiple roles in the pathogenesis of beta cell destruction leading to IDDM. Infection of the beta cell with virus may cause death of some beta cells directly but perhaps more importantly causes enhanced expression of the class 1 MHC proteins and possibly induction of the class 11 MHC proteins on surviving beta cells. The presence of this altered phenotype in genetically susceptible individuals may predispose to the development of anti-beta cell immunity with the infiltration of the islets by activated lymphocytes and macrophages. IFN-γ and TNF-α produced by activated immunocytes could further augment MHC protein expression by beta cells, resulting in enhanced reactivity with and destruction by T cells and NK cells and could inhibit beta cell function and mediate beta cell death directly.

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