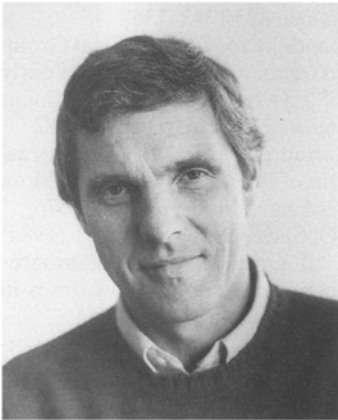


Jørn Nerup Minkowski Award, 1978, Zagreb



Dr. Nerup obtained his M.D. from the University of Copenhagen in 1964. He completed postgraduate education in Copenhagen, Denmark, the Karolinska Hospital, Stockholm, Sweden, and the Royal Hammersmith Hospital, London, UK. Dr. Nerup obtained specialist degrees in internal medicine (1976) and endocrinology (1984). Since 1976 Dr. Nerup has been Chief Physician at the Steno Diabetes Center, Gentofte Denmark. Between 1989 and 1990 he was Director of the Hagedorn Research Laboratory. Dr. Nerup is Director of the WHO Collaborating Center, Steno Diabetes Center/Hagedorn Research Laboratory for Training in Diabetes Research. Dr. Nerup is currently Professor at the University of Copenhagen. Dr. Nerup has more than 300 publications in the fields of endocrinology, diabetes and immunology. In 1992 Dr. Nerup was chairman of the WHO Study Group on the Prevention of diabetes mellitus.

On the pathogenesis of IDDM

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Summary A model of the pathogenesis of insulin-dependent diabetes mellitus, i.e. the initial phase of beta-cell destruction, is proposed: in a cascade-like fashion efficient antigen presentation, unbalanced cytokine, secretion and poor beta-cell defence result in beta-cell destruction by toxic free radicals (O_2^- and

nitric oxide) produced by the beta cells themselves. This entire process is under polygenetic control. [Diabetologia (1994) 37 [Suppl 2]: S82–S89]

Key words Insulin-dependent diabetes mellitus, free radicals, cytokines, beta-cell destruction.

Since the first experimental demonstration in 1971 of autoimmunity in IDDM [1] and of the HLA Class I-IDDM association in 1974 [2] and a year later of the

HLA Class II-IDDM association [3] more than 5200 papers on immunology and genetics of IDDM in spontaneous animal models and in man have appeared in the literature.

In the 1974 [2] paper the first comprehensive hypothesis concerning the aetiology and pathogenesis of IDDM was presented: "One or more immune-response genes associated with HL-A8 and/or W15 might be responsible for an altered T-lymphocyte response. The genetically determined host response could fail to eliminate an infecting virus (coxsackie virus B4 and others) which in turn might destroy the pancreatic beta cells or trigger an autoimmune reac-

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Abbreviations: IDDM, Insulin-dependent diabetes mellitus; TNF, tumour necrosis factor; IL-1, interleukin-1; BB, bio-breeding; NOD, non-obese diabetic; IFN, interferon; APC, antigen presenting cells; mn SOD, manganosuperoxide dismutase; NO, nitric oxide; NOS, nitric oxide synthases; ONOO⁻, peroxyxynitrite.

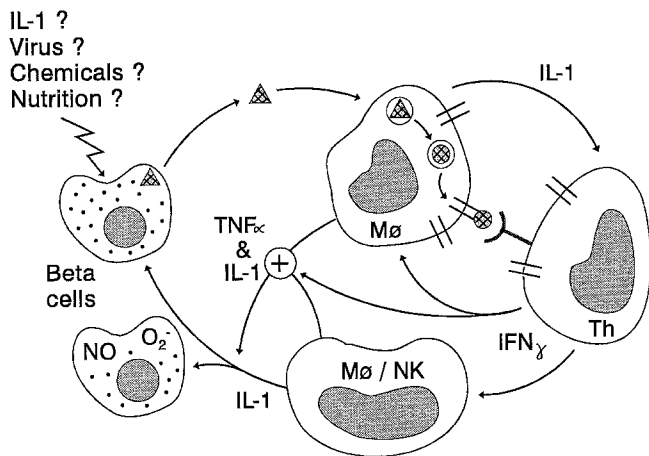


Fig. 1. Pathogenetic model of IDDM

tion against the infected organ”. On the basis of the flow of new information this hypothesis in 1981 was modified to:

“Genetic predisposition (susceptibility) to IDDM is conferred by two genes on chromosome 6, one associated with HLA-D/DR3, one with HLA-D/DR4. The reaction of susceptible individuals to certain environmental stimuli (beta-cell cytotoxic virus? beta-cell cytotoxic chemicals?) is abnormal, leading to: beta-cell destruction directly, through “autoimmune mechanisms” or because of lack of regeneration of the beta cell after damage.”

This hypothesis rapidly, however rather uncritically, gained wide acceptance, and it has almost become *dogma* that IDDM is a classic organ-specific autoimmune disease, in which beta cells are destroyed by T-lymphocyte mediated mechanisms and circulating autoantibodies are markers of the ongoing disease process. Evidence to support the participation of classic immune mechanisms in beta-cell destruction is plentiful [4–6]. However, if the term *pathogenesis* defines the earliest events and mechanisms involved in beta-cell destruction, there is ample evidence to support, in spontaneous animal models of IDDM and probably in pre-diabetic man, that a series of distinct events take place well before the invasion of the islet by T lymphocytes and beta-cell destruction by cytotoxic T lymphocytes is possible [7–10]. In other words: beta-cell destruction, hence the pathogenesis of IDDM, comprises two phases. i) a non-lymphocyte-dependent initial phase and ii) a T-lymphocyte mediated amplification and perpetuation phase of beta-cell destruction.

The initial phase of beta-cell destruction: the pathogenesis of IDDM

Based upon our findings, that supernatants of stimulated, non-diabetic human peripheral blood mononuclear cells were cytotoxic to rat and human beta

Table 1. Candidate antigens in IDDM

- Insulin/proinsulin
- 38k insulin secretory granule membrane protein
- Carboxypeptidase H
- p 52 rat insulinoma cell membrane protein
- 155 kD rat insulinoma cell membrane protein
- Gangliosides
- GAD 65 kD and 67 kD
- 37/40 kD tryptic fragment of 64 kD non-GAD islet protein
- 32, 55–72, 120–170 kD islet proteins
- GLUT 2
- Peripherin (58 kD)
- Heat shock protein (HSP) 62 and 65
- Bovine serum albumin (BSA)
- ABBOS (linear BSA epitope)
- p 69 BSA homologous peptide
- Sulphatides

cells in vitro [11, 12], the identification of IL-1 – potentiated by TNF alpha – as the cytotoxic principle [13, 14] and reviews of the literature [6, 15], we proposed a model of the pathogenesis of IDDM (Fig. 1) – the Copenhagen model.

This model gives a pivotal role to the macrophage as an antigen presenter, an accelerator of the immune response and as the producer of the beta-cell cytotoxic molecules, i. e. cytokine secretion.

Little, if any, evidence contradict the model. In fact, several groups confirmed the beta-cell cytotoxicity of cytokines [16–18]. Sequential, morphological studies of the cellular infiltrate of the islets of Langerhans in the BB rat [7, 19] and the NOD mouse [8] demonstrated that cells of the monocyte macrophage dendritic (MO/MØ/DC) lineage are the first on stage, and such cells in situ have been shown to contain abundant mRNA for IL-1 and TNF [20, 21].

Ablation experiments with silica [22, 23] showed that functional macrophages are obligatory for the disease process, whereas passive transfer experiments of spleen cells and specifically reactive T-cell clones to young NOD mice and young NOD/Scid mice suggested, that CD8+ cytotoxic T-lymphocytes are not necessary and CD4+ lymphocytes not sufficient to produce IDDM in the recipients [24].

Furthermore, the very long list of putative beta-cell specific and non-specific “autoantigens” (Table 1) identified by the presence of “autoantibodies” in IDDM sera, is difficult to reconcile with a primary and specific, T-lymphocyte-dependent antigen-driven beta-cell destructive insult.

To us this suggests that beta cells are not destroyed because islet proteins are antigens in their native form. Rather, these proteins become antigens when and because beta cells are destroyed. This means that “autoantibodies” are secondary in importance and time in the pathogenetic process and that the islet proteins are probably made antigenic through a common mechanism.

Table 2. Some important islet cell parameters affected by interleukin 1 in vitro

<i>Biphasic dose/time-dependent effects on:</i>	
insulin release; synergy with TNF-alpha	
insulin content	
glucagon release and content	
proinsulin biosynthesis	
preproinsulin mRNA	
total protein biosynthesis	
thymidine incorporation	
glucose oxidation; synergy with TNF-alpha (only rat islets)	
late Ca ²⁺ influx	
phosphoinositide metabolism	
<i>Decreases in:</i>	
aconitase activity	
ATP content	
oxygen consumption rate	
<i>Increases in:</i>	
intracellular free sodium	
prostaglandin E ₂	
heat shock protein synthesis	
c-fos protooncogene expression	
MnSOD activity	
NO synthesis	
Selective beta-cell lysis	

Thus, by definition, this mechanism – beta-cell destruction induced by IL-1 potentiated by TNF, IFN and possibly other cytokines – is in operation prior to the involvement of the “classic” immune system.

We hypothesize that understanding the molecular details of IL-1-induced beta-cell destruction equals understanding the pathogenesis of IDDM and may provide the basis for rational intervention modalities and hopefully the prevention of IDDM.

The model

The model (Fig. 1) predicts that anything from the external or internal environment which can destroy a beta cell (nutrients, virus, chemicals, IL-1) will lead to the release of beta-cell *proteins*. These proteins will be taken up by residing *antigen presenting cells* (MØ, MO, DC) in the islets, will be processed to antigenic peptides and as such be presented by MHC-class II molecules on the cell surface.

This activates the antigen presenting cells to produce and secrete monokines (IL-1, TNF) and co-stimulatory signal(s) which if T-helper lymphocytes with receptors specifically recognizing the antigenic peptide are present in the islet, induce the transcription of a series of lymphokine genes.

One of these, IFN, will feedback-stimulate the antigen presenting cells to increase expression of MHC-class II molecules and IL-1 and TNF. In addition, other cells of the MØ/MO/DC lineage present in the islet are also induced to secrete monokines.

IL-1, potentiated by TNF and IFN is cytotoxic to beta cells through the induction of free radical formation in the islet (see further).

As part of the beta-cell destructive mechanism some beta-cell proteins are damaged (“denatured”) by free radicals and in more antigenic forms (linear epitopes) presented to the immune system, thereby closing the loop in a self-perpetuating and self-limiting fashion.

The magnitude of beta-cell destruction will depend upon a) the velocity of the feed-back circuit between the antigen presenting cells and the T-helper lymphocyte, i. e. on the efficacy of antigen presentation/recognition, b) the magnitude of cytokine production, and c) on the capacity of beta-cell defense mechanisms during cytokine exposure.

Each specific element of the pathogenetic process is under genetic control, i. e. IDDM is a polygenic disorder (see further).

The model has important implications, since it eliminates the need for the existence of specific environmental trigger(s), IDDM-specific genes or gene mutations, and targetted exposure of beta cells to the cytotoxic action of monokines.

Further, it offers an explanation for the existing multitude of “autoantibodies” in newly-diagnosed and pre-diabetic IDDM: proteins released during beta-cell destruction by free radicals, may not be released in their native confirmation, but rather in free radical modified (“denatured”) more antigenic forms.

Against this background we postulate that the remarkable specificity of cellular destruction in the islets in IDDM is due to an inherent vulnerability of beta cells to cytokine-induced free radical damage. In other words: the beta cells die because they are beta cells.

IL-1 effects on islets in vitro and in vivo: Exposure of rat islets in vitro to IL-1 (recombinant human IL-1β 150 pg/ml, specific activity ~ 400 U/ng, in RPMI 1640 for 24 h) has dramatic effects on beta-cell function and islet morphology (Table 2, Fig. 2).

Longer-term exposure (5–7 days) completely disintegrates islets and destroys beta cells as well as alpha cells. The shorter term exposure does not affect alpha-cell function or morphology, but induces severe inhibition of insulin release and heavy damage to beta cells ranging from extensive degranulation to complete cytolysis [25].

Repeated exposure alternating with 5-day recovery periods in IL-1 free medium, has increasingly devastating effects on insulin secretion and its recovery. Glucagon secretion, however, is unaffected by repeated IL-1 exposure [26]. These in vitro findings were confirmed in experiments on the intact pancreatic gland in situ [27] and a diabetes-like condition characterized by hyperglycaemia and hypoinsulin-

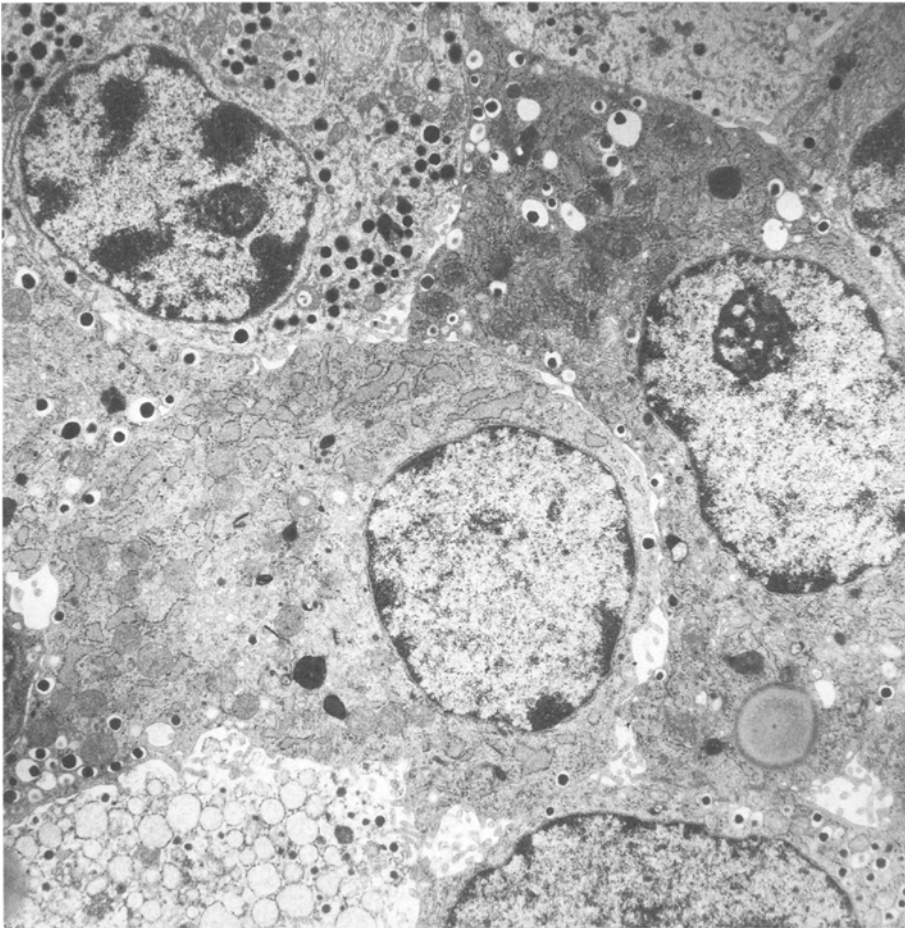


Fig. 2. Electron microscopy photograph ($\times 5000$) of a rat islet of Langerhans showing severe beta-cell damage (lower left) and moderate beta-cell damage (right) and intact alpha cell (upper left)

aemia is seen in normal rats receiving daily IL-1 injections ($4 \mu\text{g}$ per kg body weight) i.p. for 5 days [28]. Mouse islets in culture are more resistant to IL-1 cytotoxicity than rat islets. By increasing the low calcium concentration (0.42 mmol/l) of RPMI 1640 to near physiological concentrations, the IL-1 sensitivity of mouse islets becomes similar to that of rat islets [29]. Few studies of IL-1 effects on human islets in culture have been reported and with conflicting results. Almost complete resistance [30] and “normal” sensitivity i.e. similar to the islets, [31] have been found. More studies are required to solve this controversy and to reveal the reasons behind strain [32], species and possibly individual differences in beta-cell sensitivity to IL-1 cytotoxicity.

IL-1 induced beta-cell cytotoxicity in vitro is dependent upon energy generation, DNA synthesis and metabolic activity of the beta cell [33, 34]. This led us to suggest that the beta cell may be seen as a “moving target”. The “resting” beta cell is more resistant than the “active” beta cell, e.g. low glucose, amiloride and somatostatin shift the dose-response curve to the right; high glucose and leucine in the culture medium to the left [33–35].

The molecular mediators of IL-1 induced beta-cell destruction

The IL-1 induced phenomena listed in Table 2 all take hours to occur. Searching for a common, rapidly acting, mechanism capable of explaining the selectivity of beta-cell destruction as well as the diverse phenomena in Table 2, we suggested a role for free radicals first in the form of superoxide anions O_2^- [36]. This concept was supported by a series of observations. In brief: IL-1 activates the Na^+/H^+ (antiport) system [35], which may lead to O_2^- formation in the mitochondria as seen in other tissues [37].

IL-1 induces an IL-1 and islet specific oxidative stress protein (alternative defense against O_2^-) response in islets in vitro [38]. An identical response could be induced by islet exposure to H_2O_2 [38]. Beta-cell free radical scavenging potential is low, due to low content of MnSOD [39]. O_2^- scavenging molecules protect beta cells against cytokine induced cytotoxicity [40].

Accordingly, beta cells are not exposed to specific cytotoxic mechanisms. IL-1 (and TNF) may also in non-beta cells induce formation of O_2^- as well, the effects of which are neutralized by activation and increased expression of scavenger enzymes (e.g.

MnSOD) for which IL-1, potentiated by TNF, is the natural inducer. Thus, in all IL-1 receptor-bearing cell types, IL-1 initiates a kind of race between protective and deleterious events. In beta cells the deleterious prevail. Consequently, we hypothesize that beta cells are exquisitely IL-1 sensitive because of their low content and inducibility of the scavengers.

Our recent *in situ* hybridization experiments support this by showing that only moderate (less than two-fold) increases in expression levels of MnSOD and haeme oxygenases 1 and 2 are induced by IL-1 in rat islets during 24- to 48-h incubation periods (Cuartero, unpublished observation).

The observation by Southern et al. [41] that islets exposed to IL-1 release nitrite as an indication of the induction of another free radical species NO, and the demonstration [42], that NO produced chemically is cytotoxic to islet cells further substantiates a role for free radicals in beta-cell destruction.

NO is produced during the conversion of L-arginine to citrulline by a family of enzymes, the nitric oxide synthases (NOS). Three isoforms of NOS exist, two constitutive forms (cNOS) cloned from brain and endothelial cells (both Ca^{2+} dependent, produced in small amounts for short periods of time) and one inducible form (iNOS) which is Ca^{2+} independent and produced in large amounts for longer periods of time in macrophages, hepatocytes, smooth muscle cells and endothelial cells [43].

cNOS and iNOS have about 65% sequence homology. The sequences of the iNOSs are identical within and almost identical between species (mouse/rat). Although structurally identical the iNOSs require different inducers for their expression, e.g. endotoxin and IFN on macrophages [44], and IL-1 on chondrocytes and smooth muscle cells [45, 46]. This suggests, that inducer-specific signal transduction pathways and/or DNA regulator sequences exist in the genes in different iNOS producing cell types. Rat islets cultured in RPMI 1640 show no spontaneous iNOS synthesis as evidenced by mRNA and nitrite release ($< 20 \text{ pmol}$). However, when exposed to IL-1, mRNA is detectable after 30–60 min. (Cuartero, unpublished observation) and nitrite release is seen after 8–10 h, continuing for 24 h or more to impressive levels (approximately $10 \text{ pmol} \cdot \text{islet}^{-1} \cdot 24 \text{ h}^{-1}$) [41, 47].

From IL-1 stimulated rat islets we have cloned an iNOS with a coding sequence nearly identical to that of the other rat iNOSs [48]. This iNOS can be induced by IL-1 in RIN-cells and by a mixture of cytokines (IL-1, TNF, IFN) in human islets [47] as measured by nitrite release to the tissue culture medium. The cloning and sequencing of the human islet iNOS is in progress. The iNOS gene has been mapped to mouse chromosome 11 (syntenic regions on chromosomes 10 and 17 in rat and man respectively) [49].

It has been a matter of debate in which cells in the islets NO is produced. The Düsseldorf group [50] maintains that NO is released by macrophages and endothelial cells, whereas we and the St. Louis group [51] see the beta cell itself as the major site of production because: 1) in islets exposed *in vitro* to NO produced by chemical NO donors both alpha- and beta-cells are destroyed [52]. 2) Human macrophages are still to be shown capable of producing NO. 3) IL-1 induces iNOS synthesis in sorted beta cells, but not in alpha cells [53].

However, in the intact islet of Langerhans *in vivo* during the disease process, most likely beta cells and endothelial cells as well as macrophages are involved as NO producers and thereby in beta-cell destruction.

The involvement of iNOS synthesis in beta-cell destruction explains the need for energy generation and protein synthesis during the process of beta-cell destruction. In fact, more than 25 islet proteins are up-regulated during IL-1 exposure [47]. The finding that increases in glucose concentrations lead to upregulation of iNOS expression (Andersen, unpublished observation), supports the moving target concept. Little is known, however, about the regulation of iNOS gene expression and such studies are clearly in demand. iNOS expression has also been demonstrated in islets of diabetes prone BB-rats close to the onset of diabetes [54].

Although a major player, NO formation alone cannot fully explain the devastating effects of IL-1 exposure on beta cells. Rat islets cultured in arginine-free RPMI 1640 produce no NO when stimulated with IL-1. Nevertheless, the initial increase in insulin release is preserved, and the later decrease of insulin release is inhibited by only about 50% [41, 55], supporting the existence of an NO-independent (O_2^- dependent?) pathway of IL-1 effects on beta-cell function and survival.

The molecular mechanisms of IL-1 induced, free radical mediated beta-cell destruction

Beta cells do express IL-1 receptors [31, 56], but very little is known about receptor signalling pathways in any IL-1 responsive cells. Hence, how IL-1 exposure alone (or in combination with TNF and IFN) leads to alterations in gene-expression, notably of insulin and iNOS, is not known at present. How the mediators of IL-1 induced beta-cell destruction, the free radicals O_2^- and NO^- , may be generated, we have attempted to describe above. The consequences of their occurrence are shown in a very simplified schematic form in Figure 3.

Free radicals are known to produce DNA strand breaks [41, 57] to be followed by poly(ADP)ribose polymerase activation and NAD depletion resulting in cell death. NO has been demonstrated by electron

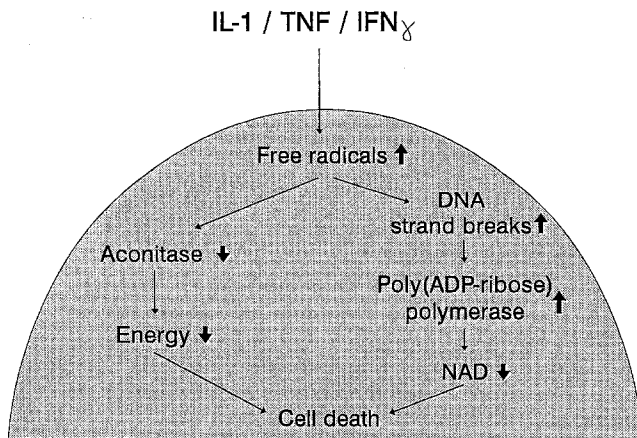


Fig. 3. Schematic model of cytokine-induced molecular mechanisms of beta-cell destruction

paramagnetic resonance to form iron-nitrosyl complexes [58]. This will inactivate important enzymes, e.g. aconitase, which IL-1 has been shown to do [59]. The consequence is decreased ATP generation and subsequent cell death.

In addition NO and O_2^- may modify other protein functions by nitrosylation and oxidation.

When not scavenged, due to low beta-cell scavenging potential, O_2^- may react with NO to form the powerful oxidant peroxynitrite ($ONOO^-$) a source of the very toxic hydroxyl (OH^-) free radical [60].

$ONOO^-$, and probably also NO and O_2^- may “denature” beta-cell proteins. Finally, $ONOO^-$ may react with metalloproteins, e.g. CuZnSOD [61], to form a nitronium-like intermediate, which nitrates tyrosine residues on intracellular signalling molecules such as tyrosine kinases.

This should provide mechanisms enough to explain beta-cell destruction during IL-1 exposure. It may further explain how beta-cell proteins become beta-cell antigens through a common mechanism because and when beta cells are destroyed. We suggest, that this is what triggers the T-lymphocyte dependent amplification and perpetuation phase of the pathogenetic process leading to IDDM.

The process of cytokine-induced beta-cell destruction is, however, probably rather more complicated than suggested in this paper. IL-1 induces the upregulation of more than 25 islet proteins as demonstrated by 2D-SDS gel studies [47]. When islets are protected against IL-1 cytotoxicity, e.g. by nicotinamide [55], arginine free culture conditions [41, 55] or by L-arginine analogues the number of protein changes is considerably reduced.

The pathogenetic model (Fig.1) implies that IDDM in man is a highly polygenic disorder. The polygenic nature of IDDM is supported by findings in the NOD mouse [62] and the BB-rat [63]. We have proposed [reviewed in 10], that in human IDDM common alleles of normal genes recurring in

unfavourable combinations confer susceptibility to IDDM by encoding a complex phenotype characterized by i) efficient antigen presentation (HLA-class II), ii) unbalanced cytokine secretion and iii) poor beta-cell defence mechanisms.

We expect that the identification of these genes, their corresponding proteins and their functions will finally lead to the full understanding of beta-cell destruction at the molecular level and in the end rational approaches to the prevention and cure of IDDM.

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