DENDRITIC CELLS AND MACROPHAGES IN INSULIN DEPENDENT DIABETES MELLITUS

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DENDRITIC CELLS AND MACROPHAGES IN INSULIN DEPENDENT DIABETES MELLITUS

DENDRITISCHE CELLEN EN MACROFAGEN IN INSULINE-AFHANKELIJKE DIABETES MELLITUS

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Science is a first-rate piece of furniture for a man's upper-chamber, if he has common-sense on the ground floor

O.W. Holmes
The Poet at the Breakfast-Table

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Chapter 1

GENERAL INTRODUCTION

1.1 Diabetes mellitus, the clinical picture

The onset of diabetes mellitus is characterized by various symptoms, all the result of a disturbed glucose metabolism. The main symptoms are thirst and polydypsia, polyuria, glucosuria, and weight loss. The faster the onset of diabetes, the more prominent these symptoms will be.
The disturbed glucose metabolism underlying these symptoms, is due to an absolute deficiency of insulin secretion, a reduction in its biologic effectiveness or both.
Diabetes mellitus is nowadays classified in two major types:
Type 1, or insulin-dependent diabetes mellitus (IDDM), occurs most commonly in juveniles. It is a catabolic disorder in which circulating insulin is virtually absent, since the pancreatic β-cells, due to their absence, cannot respond to any insulinogenic stimulus. Administration of exogenous insulin is therefore required to reverse the catabolic state, to prevent ketosis and to bring the elevated blood glucose level down.
Type 2, or non-insulin-dependent diabetes mellitus (NIDDM), represents a group of milder forms of diabetes that occur predominantly in adults. Circulating endogenous insulin is almost always sufficient to prevent ketoacidosis, but is often either subnormal or relatively inadequate in the face of the increased needs owing to tissue insensitivity.

The longer diabetes mellitus exists, the greater the chance of developing the characteristic complications: micro- and macroangiopathy, and neuropathy. Microangiopathy may cause retinopathy and nephropathy, while macroangiopathy gives rise to an elevated incidence of coronary heart disease, cerebro-vascular accidents, and ischemic complications in the legs. The quality of life of diabetic patients can be profoundly reduced by these complications, while also the life-expectancy is considerably shorter for diabetic patients.
Therefore, prevention of late complications, and prevention of the disease itself, are major goals in diabetes research. Prevention of the disease can only be expected if the mechanisms leading to the disease will be better understood. Though some studies have indicated that some type 2 patients show characteristics of type 1 disease[1,2], and that the boundaries between type 1 and type 2 diabetes might be not as absolute[3], it is nevertheless generally assumed that the mechanisms leading to either type 1 or type 2 diabetes are different.

This thesis considers aspects of the mechanism leading to IDDM or type 1 diabetes mellitus.

1.2 Epidemiology of IDDM

The incidence of IDDM shows considerable variations between different areas. There appears to be a more than 35-fold difference in the risk of developing IDDM between countries with the highest incidence of the disease (Finland, 28.6/100,000/year) compared to countries with the lowest incidence (Japan, 0.8/100,000/year)[4]. In the Netherlands the incidence is 10.9/100,000/year. Many variables have been tested for association with these differences in incidence. Among them, the distance to the equator appeared to show the strongest association ($r = 0.76$). Sardinia is a striking exception to this rule. Etiologic explanations that have been proposed for this geographic association, are genetic variations, influence of temperature, differences in diet, and contributions from hygienic circumstances.

Genetic associations appear to play an important role in type 1 diabetes mellitus, though it has become clear that only the susceptibilty for the disease, and not the disease itself is inheritable: monozygotic twins are concordant in only 30-55% of the pairs[5,6]. The strongest genetic associations are described for alleles linked to immune function, for example the MHC-class II genes located on chromosome 6, and the transporter genes *Tap-1* and *Tap-2*, closely linked to the MHC-class II genes[7], which play a major role in the delivery of endogenous peptides to the endoplasmic reticulum. In Caucasians the DR3 and DR4 alleles of the HLA-DR gene confer a high relative risk for IDDM[8,9], while the presence of the DR2 allele appears to protect from developing IDDM[10]. It has become clear that this resistance to diabetes is more closely linked to the presence of DQw8, in linkage disequilibrium with DR2[11]. Todd found that the presence of aspartic acid at position 57 of the DQβ chain was strongly associated with protection from diabetes: the occurrence of DQβ alleles without aspartic acid on position 57 was significantly higher in the diabetic population[12]. The combination of a non-asp amino-acid on DQβ-57, with an arginine residue on position 52 of the DQα chain appeared to confer the highest risk for diabetes in Caucasians[13].

Apart from the MHC-region, polymorphisms of the region flanking the 5'-end of the insulin gene on chromosome 11 have been shown to be associated with the disease[14]. Also, genetic abnormalities of β-cell metabolism might be important for the development of diabetes, since discordant identical twins and first degree relatives of type 1 diabetic patients are described to have an elevated fasting proinsulin level[15,16].

Furthermore, the sex of the individuals appears to play a role. In the USA, the relative frequency in females at the onset of puberty is higher than in males[4], though in Japan this is described to be the other way around.

1.3 The early evidence that IDDM is an autoimmune mediated disease

The first suggestion of the involvement of the immune system in the development of IDDM dates from 1965, and came from the histopathologic examination of pancreases from young diabetic patients, part of whom died within six months after the onset of the first symptoms. In some of these patients inflammatory infiltrates of mononuclear cells around or in the islets of Langerhans could be demonstrated[17]. Using the more discriminative technique of immunohistochemistry on paraffin embedded material collected during the previous 25 years from patients who died from diabetes, Foulis confirmed that these infiltrates were of leucocytic origin, and that the number of insulin containing islets was dramatically reduced[18]. Bottazzo et al demonstrated the presence of $CD4^+$ and $CD8^+$ T-cells in these mononuclear infiltrates around the islets, intermingled with a few IgG synthesizing B-cells[19].

Another parameter indicating the nature of the disease mechanism was the demonstration of islet cell antibodies (ICA) in the blood from patients suffering from poly-endocrine autoimmune disease[20]. Shortly thereafter ICA were described in the sera of newly diagnosed diabetic children[21]. Depending on the study and on the detection technique used, 50-90% of the IDDM patients showed ICA-positivity at the onset of the disease[22,23,24]. In the majority of the patients ICA disappeared within 6 months to 3 years after clinical onset of the disease[21]. Screening of first-degree relatives for ICA revealed that ICA may be present up to 8 years before clinical onset of diabetes[25], indicating that IDDM is an indolent disease process whose underlying pathogenetic mechanisms may progress slowly over long periods of time.
The nature of ICA antigen(s) is not known, but several candidates have been put forward. These include an unidentified protein of 38kD[26], another protein of 39kD[27], a constituent of the β-cell granules (carboxypeptidase-H[28]), and the formerly described 64kD antigen.This latter antigen appeared to be an isoform of the enzyme glutamic acid decarboxylase (GAD)[29], of which at least two forms exist, GAD65 and GAD67[30]. GAD65 is abundant in islets and is believed to represent the 64kD antigen[30]. Also antibodies to insulin have been observed in IDDM, often before clinical onset of diabetes occurred[31].

In conclusion, the histology of pancreases of patients with recent onset diabetes, and the presence of antibodies to β-cell related antigens often long before the onset of clinical diabetes suggest that IDDM is an autoimmune disease.

1.4 Animal models for spontaneous IDDM, in particular the NOD mouse

Research on patients has made clear that type 1 diabetes mellitus is associated with a β-cell directed autoimmune reaction. However, these studies could not make clear how such an autoimmune reaction develops. Since it is difficult to identify pre-diabetic individuals with certainty, and even more difficult to investigate their pancreas without invasive techniques, it was necessary to establish experimental animal models of insulin-dependent diabetes mellitus to elucidate the exact mechanisms of the β-cell directed autoimmune reaction.

In the mid-seventies two animal models of spontaneous autoimmune mediated type 1 diabetes were established, namely the BB-rat and the NOD-mouse.

The BB-rat was bred from the Wistar-rat, and develops type 1 diabetes mellitus in varying frequencies depending on the subline and on the breeding facilities. The incidence of diabetes is about the same for male and female rats from the same colony. Diabetes in the BB-rat is autoimmune mediated and is associated with a progressive lymphopenia[32], particularly reflected in a deficiency of $RT6^+$ T-cells, which have been described to have a immuno-regulatory function[33].

The NOD mouse was established from a spontaneously diabetic female mouse during inbreeding of the CTS strain[34]. Inbreeding of the strain was performed for twenty generations before distribution of the mice was allowed. Nevertheless, there now appear to be large differences in diabetes frequency and time of onset between the various NOD-colonies in the world[35]. Although environmental factors such as diet[36], temperature[37], and virus exposure[38,39] have been considered as causing these differences in the incidence of diabetes, their precise contributions are still not known. Notwithstanding the large variations in overall incidence in the different colonies, all colonies show a significantly higher incidence of diabetes in their females as compared to their males[35]. If NOD mice are gonadectomized, a slight reduction in the incidence is observed in females, whereas in males a considerable rise in the incidence is found, reaching incidences comparable to intact females of the same colony[40,41].
The infiltration of leukocytes around and into the islets, generally called peri-insulitis and insulitis respectively, is the most prominent histopathologic feature of NOD mouse diabetes[34]. Both sexes show these infiltrates from 4-5 weeks of age onwards[40]. The infiltrates initially aggregate around the perimeter of the islets (peri-insulitis). Later the cells infiltrate the islets, and β-cell loss can be observed. The presence of antibodies on β-cells prior to the development of insulitis has been documented in the NOD

mouse[42], and also the presence in serum of anti-GAD[43], anti-insulin[44] and anti-thymocytotoxic[45] antibodies has been described.
Apart from insulitis and diabetes, NOD mice also spontaneously develop sialoadenitis and infiltrates in the lacrimal glands[46]. Thyroiditis can be induced in NOD-mice by modulations in the iodine content of the diet[47].
The development of diabetes mellitus in NOD mice is influenced by many susceptibility genes, 10 of which are now mapped[48], and of part of them the probable function has been identified. The most important genes that are likely involved are the gene encoding for the unique IA antigen in the MHC-class II of the NOD mouse, possibly the structural gene for IL-2[48], and the gene encoding for the high affinity receptor for IgG[49]. Also the Bcl-2 gene and the gene for the IL-1 receptor are located in areas to which diabetes susceptibility genes have been mapped[50].

In the NOD mouse not only the mechanisms leading to the disease have been investigated, but also various treatments which might prevent the development of diabetes.
The first therapeutic option, based on the assumption that diabetes type 1 is an autoimmune disease, was immunosuppression. As expected, compounds like Cyclosporine A, effectively prevent diabetes in the NOD mouse[51,52]. Though Cyclosporine A is described to prolong the honeymoon period in newly diagnosed diabetic patients, the use in healthy individuals at high risk of developing diabetes is not advocated, since the adverse effects, like potential kidney damage, or a higher incidence of neoplasms, are too serious[53].
Immunoregulation is another option. Favorable outcomes have been obtained with cytokine treatments in the NOD mouse and the BB rat: IL-2[54], IL-4[55] and IL-10[56], but also IL-1[57], and TNF-α[58,59] have been shown to be effective in the prevention of diabetes. An additional prospect of regulating the immune response was adjuvant therapy or BCG-vaccination, as a general stimulator of the immune system. This treatment appeared to be effective in NOD mice[60], and recent clinical trials have also indicated its possible effectiveness in the human situation[61]. Another immunoregulatory intervention is injection of islet-antigens into the thymus. Injection of GAD65 or syngeneic islets into the thymus induced transferable T-cell tolerance in NOD-mice[43]. Comparable tolerization procedures in humans have not yet been investigated.
Based on the observation that shortly after the start of insulin therapy in newly diagnosed patients, a period of reduced or absent need for exogenous insulin often occurs, preventive therapies of insulin administration were started in NOD mice and in BB rats[62,63]. Since these strategies were effective and the expected negative side-effects were not serious, a preventive insulin administration trial has been set up in pre-

diabetic patients. The first results are promising[64].

In conclusion, the histologic and immunologic data show that the NOD mouse and the BB rat can be used as animal models for human IDDM. Though human IDDM may be heterogeneous in respect to its pathogenesis, the mechanisms of the disease can at least partially be clarified by studying the NOD mouse and BB rat. These studies might also provide new perspectives in the prevention and treatment of human type 1 diabetes.

1.5 Evidence that T-cells are essential in the development of IDDM

Immunohistologic examination of the islet-inflammatory lesions reveal that the majority of the lymphocytes around the islets are T-cells, both in the human[19], as well as in the BB rat and the NOD mouse[65].
Their functional importance in the development of the disease was proven by the finding that in neonatally thymectomized BB rats[66] and NOD mice[67], and in athymic nude NOD-mice, which lack T-cells[68], diabetes did not occur. The recently developed NOD-*scid/scid* mouse, lacking both T- and B-cells is also free of diabetes[69].
Early transfer studies from diabetic donors to non-diabetic acceptors made clear that diabetes is most effectively transferred with a combination of $CD4^+$ and $CD8^+$ T-cells[70,71]. However, studies have also been described showing an acceleration of diabetes upon transfer of a $CD4^+$ T cell clone, without co-transfer of $CD8^+$ T-cells[72]. Due to a possible recruitment of T-cells of the other subset in the acceptor, it could not be concluded that the transfer of diabetes with $CD4^+$ cells only is in fact possible. The recent development of the NOD-*scid/scid* mouse solved this problem. Using monoclonal antibodies to prevent outgrowth of contaminating donor $CD8^+$ T-cells in the acceptor, it was demonstrated that transfer of $CD4^+$ T-cells alone gave rise to insulitis, but not to β-cell destruction and diabetes. Transfer of $CD8^+$ T-cells only induced neither insulitis nor diabetes. If $CD8^+$ T-cells are transferred together with or after $CD4^+$ T-cells, insulitis and β-cell destruction does occur[73].

$CD4^+$ T-cells can be subdivided by differences in cytokine production, the two extremes of the scale being Th1 and Th2 cells[74,75]. Th1-cells characteristically produce IL-2, IFN-γ, and TNF-β, whereas Th2-cells secrete IL-4, IL-5, and IL-10. The functional significance of these different cytokine profiles is that they lead to different T-cell actions. Th1 cells and their cytokine products lead to cell-mediated immunity (DTH reactivity and macrophage mediated responses), while Th2 cells are more

effective in supporting the humoral immune response. Among signals that may orient the immune response in the direction of either a Th1- or a Th2-response, the macrophage-derived cytokines, IL-10 and IL-12 have been discovered to play important roles. IL-12 is a potent stimulator of Th1-cells and their cytokines[76,77]. IL-10, in contrast, inhibits the production of IL-12[78,79]. It also exerts anti-inflammatory effects by increasing IL-1 receptor antagonist and inhibiting the generation of oxygen and nitrogen free radicals. The combination of IL-4 and IL-10 is particularly effective in inhibiting Th1 effector function[80].
The choice into the direction of a more Th1- or a Th2-like response can also be influenced by metabolites of the arachidonic acid metabolism. Prostaglandin E (PGE), produced by macrophages and other cells (e.g. fibroblasts) decreases the production of IL-2 and IFN-γ by the Th1 subset[81,82,83]. There is no effect on IL-4 production[82,83], but the IL-5 secretion is enhanced[82]. Thus, production of PGE by macrophages is thought to result in a stimulation of Th2-cells at the expense of Th1.
Whereas PGE is directing the immune response into the direction of Th2, another metabolite of the arachidonic acid metabolism, leukotriene C4 is described to have a stimulatory effect on the production of IFN-γ[84].
Evidence is accumulating that the type of cytokines, produced in an insulitis process influences the β-cell destructive capacity of the infiltrate. If cytokines are produced that are particularly compatible with a Th1 profile (IFN-γ), β-cell destruction occurs and diabetes will develop[85,86]. If, on the other hand, IL-4 and IL-10 are produced, there is protection from β-cell destruction and diabetes[87,88].

In conclusion, T-cells are essential in the development of IDDM. Cytokine profiles of the T-cells involved modify the β-cell destructive capacity of the insulitis. On the one hand these cytokine profiles may be influenced by macrophage secretion-products, and on the other hand may themselves influence macrophage function.

1.6 Antigen presenting cells and the development of IDDM

1.6.1 Antigen presentation

For T cell stimulation and the generation of antigen specific T cells it is necessary that antigen is presented to T cells in the appropriate form (peptide-MHC-complex) by antigen presenting cells[89] (APC). The antigen is processed by APC into suitable peptides, that fit into the groove of the MHC-molecule in which the antigen is presented to the T cell receptor.
In the case of exogenous antigens, APC must be able to take up antigen and degrade it

into peptides. Another possibility would be to take up peptides that have already been degraded by other cells. Intracellularly, the process of combining peptides to MHC molecules is generally as follows: MHC class II molecules, assembled in the ER and transported through the Golgi to the Trans Golgi Reticulum fuse with late endosomes, where the degraded peptides are accumulated (after being taken up and processed in early endosomes). Peptides of varying length, about 14 amino acids long[90], replace the invariant chain occupying the groove of the MHC class II molecule[91,92]. How the MHC class II molecules are transported to the cell surface is still unknown. $CD4^+$ T-cells are able to recognize peptides presented in the groove of the MHC-class II molecule[90].

Endogenous antigens are probably first degraded into peptides of 8-10 amino acids[93] by the low molecular mass polypeptide (LMP)[94] complex, associated with the ubiquitin-dependent proteasome[95], present in the cytoplasm. Peptides are then delivered to the MHC class I molecules in the lumen of the endoplasmic reticulum by specialized transporters (TAP)[96,97]. After incorporation of the peptides into the groove and exposure of the MHC class I molecules on the cell surface, the $CD8^+$ T-cells are able to recognize such peptides presented in the context of the MHC-class I molecule[89,98]. Both CD4 and CD8 molecules have been shown to act as co-receptors for their respective MHC-molecules during this interaction of the TCR with the peptide-MHC complex[99].

Apart from the recognition of peptides in the appropriate MHC-molecule, a second receptor signal is required for primary T-cell activation. This is probably provided by the binding of the CD28 molecules on the T cell to its B7-2 ligand (and also B7-1, but with a lower affinity) on the APC[100]. Binding of CTLA-4 to B7-1 and B7-2 may also occur, but is probably later in the process, since CLTA-4 is primarily seen on T cells after activation[101]. If the B7 co-stimulatory signal is not provided, T-cell anergy results[102].

Before the above described cellular interaction can occur, a close physical contact between the T-cell and the APC has to arise. Such contacts are stabilized by the interaction between adhesion molecules and their ligands, such as LFA1 with ICAM1 or CD2 with LFA3. Inhibition of this process by monoclonal antibodies to either one of these adhesion molecules have been shown to inhibit the triggering of T cells[99,103].

Three different cell types are important in antigen presentation: the dendritic cells, the macrophages, and the B-cells.

In conclusion, antigen presentation is a complex process, which is required for T-cells to mount an antigen specific immune response. Three cell-types can exert an antigen presenting function: B cells, macrophages, and dendritic cells

1.6.2 Dendritic cells

Dendritic cells (DC) are unique APC in that they are the only APC that are capable to effectively stimulate naive T-cells[104,105]. The trigger for T-cell proliferation [T-cell receptor recognition of the MHC-peptide complex together with the costimulatory signal B7-1/2↔CD28[106]] requires a close physical contact of the DC with the responding T-cells. This close physical contact is in vitro detectable when DC form cellular clusters with T-cells[107,108]. An important observation in this respect is that in the mouse DC will form clusters with T cells at 37°C in the absence of antigen, whereas macrophages will only do so after exposure to antigen[109]. The cluster formation is dependent on ICAM1 and LFA1 since it is inhibited by monoclonal antibodies to these adhesion molecules[110]. It is also dependent on the active movements of the DC (37°C).

Well differentiated DC have limited phagocytic capacity. At an earlier stage, as interstitial DC or Langerhans cells (LC), they have been described as capable of taking up antigen, probably assisted by their low level expression of Fc-, complement- and/or other receptors[111,112]. A question remains as to the ability of DC to degrade ingested molecules. DC do have a lysosomal system, albeit reduced compared to macrophages. Enzymatic degradation of proteins into peptides and their association with class II molecules probably takes place in acidic lysosomes[113]. Indicative of their specialized function, DC retain antigenic peptide-MHC class II complexes for prolonged periods in culture (1-2 days)[113], whereas macrophages have a turnover measured in hours[114].

One of the major difficulties in identifying and investigating DC is the absence of a defined marker that is only present on DC, and that is present on all DC. In mice several monoclonal antibodies recognize subpopulations of DC. 33D1[115] is relatively restricted to spleen DC in the marginal zone, whereas NLDC-145[116] stains a 145kD protein that is described to be present on lymph node IDC and DC in the white pulp of the spleen. N418 recognizes CD11c, which is present in high density on murine DC[117], but also macrophages and other leukocytes express this molecule. M342 reacts with an antigen within the granules of DC[118], but also of some B-cells, and is not present in freshly isolated spleen DC. In the human situation CD1 reagents can be conveniently used for identification of LC[119]. Another marker frequently used to date is the dot-peri-nuclear staining of CD68[120], compared to the widespread cytoplasmic positivity in macrophages.

Since markers are not completely reliable to identify DC, function is another key to DC identification. Presently, a DC is functionally defined as a cell with the ability to stimulate naive T cells (a primary T-cell response), with a relatively limited phagocytic capability, and a typical motility[121]. DC actively extrude and withdraw long

cytoplasmic extensions (veils) and actively seek contact and interact with other cells, most notably T lymphocytes to form cellular clusters[108]. Morphologically, they hence have a dendritic appearance. Other concomitant identification criteria are a reniform nucleus, and a high density of membrane MHC class I and class II molecules[122,123]. Also the expression of adhesion molecules, like ICAM1[124], ICAM2[126], LFA1[126], and LFA3[124], and expression of B7/BB1[127] is a characteristic of DC.

Several different types of DC are recognized according to their histology and their functional properties.

Interstitial DC are found in virtually all organs, except in the so-called immunoprivileged sites, such as the brain, the testes, and part of the eye. They sometimes form a network, which was first described in rats[128,129], but is now also well documented in man[130]. The number of interstitial DC is influenced by genetic factors and declines with age[131]. LC in the skin also form a network and show many characteristics of the interstitial DC. Hence they are considered as DC. There are, however, also obvious differences: LC have Birbeck granules and interstitial DC do not. The function of the Birbeck granules is not known. Interstitial DC and LC carry low levels of FcγRII (CD32)[132]. Isolated LC have been shown to have antigen processing and presenting capacity and develop lymphoid DC-like properties *in vitro*[133,134]. LC and interstitial DC of the gut have been shown to migrate into the afferent lymph as veiled cells (VC) to finish as interdigitating cells (IDC) in the T-lymphoid areas of the draining lymph nodes[135]. It seems likely that DC from other organs, like the pancreas, the heart, or the kidney, may undergo similar migration under exposure to antigen or stimuli. The grouping of interstitial DC near the vessels and the draining lymphatics[129] suggests this way of exit to the draining lymph node as plausible.

Veiled cells, or afferent lymphatic dendritic cells have been isolated from lymphatics draining the tissues, and show the characteristics of dendritic cells, notably the ability to stimulate strong T-cell responses[136]. Morphologically, these cells show large veiled membrane protrusions[137,138]. Antigen loaded VC enter the T-cell areas of lymph nodes, where they probably become (IDC)[139]. IDC in the draining lymph nodes are probably the histologic counterpart of lymphoid DC isolated from human tonsils, on which many of the human functional studies of lymphoid DC have been performed. The phenotype of these IDC in tissue sections does not exactly correspond to the phenotype of isolated tonsil dendritic cells[140,141]. However, technical and activation changes probably account for these differences[139].

Lymphoid DC is a functional term, used to indicate DC that have been isolated from lymph nodes, spleen, tonsils, or blood[141,142,143]. Therefore "lymphoid DC" is overlapping with the other types of DC, which are mainly characterized by

histomorphological criteria mentioned before. *In vitro* lymphoid DC have a poor endocytotic capacity, and stain weakly for acid phosphatase. Birbeck granules are not present[122].

Another feature of the various DC is their failure to proliferate *in vitro*[122]. This and their normally low numbers in the tissues represent major obstacles for obtaining large numbers of pure DC, which has significantly hampered functional studies of DC, and the development of a unique monoclonal antibody to identify DC from other cells.
Typical DC have also been described in the thymus. Ultrastructural studies in rats have demonstrated that they occur in the largest number at the cortico-medullary junction[144,145]. Like other DC, thymic DC are also capable of stimulating T-cell responses and indeed appear to provide the stimulus for thymocyte proliferation[146].

In conclusion DC are a heterogeneous population of APC. Though heterogeneous, all these DC are superb APC and capable of stimulating naive T cells to respond. In the latter capability DC are unique.

1.6.3 Origin of dendritic cells. Methods for isolation of precursors for different types of dendritic cells

DC are bone marrow derived. Total body irradiation, with a dose that induces bone marrow destruction, causes the disappearance of interstitial DC from rat tissues. Reconstitution with autologous bone marrow results in interstitial DC to be present in normal numbers by 10 days[123,128]. Also in the human situation there is evidence that DC are replaced by cells from donor origin after bone marrow transplantation[147]. As a further confirmation of their bone marrow origin DC express CD45 common leucocyte antigen, the antigen shared by all cells from hematopoietic origin.
Precursors for DC probably leave the bone marrow via the blood, and circulate in the blood stream in small numbers, possibly on their way to the tissues.
Since peripheral blood is easily accessible, several methods have been developed to isolate DC cells or their precursors from the blood, to allow investigation of human DC. The classical way of isolating them from the blood is by the method described by Knight[148], using a metrizamide density gradient, plastic-adherence- and culture steps. Recently developed methods using cytokines like GM-CSF[149], TNF-α[150], IL-4[151], sometimes in combination with IFN-γ[152] to stimulate the differentiation of blood precursors into DC yield higher numbers of DC with a good viability and a purity that is comparable to, and often even higher than the classical method.
It is presently assumed that there might be different lineages for DC development, which may account for their heterogeneity. $CD34^+/CD1a^+$ precursor cells from bone

marrow and fetal cord blood are capable of differentiating into Birbeck granule (BG) containing DC[153], whereas $CD34^+/CD14^+$ monocytes from the same sources are able to differentiate into so-called "monocyte-derived" DC without BG. The Knight-method as it is modified by Kabel[154] using a short incubation of monocytes with metrizamide, and the later developed method by Mooij[155] using tri-iodothyronine (T_3) in the same way, act via stimulation of differentiation of monocytic precursors into DC. If such DC are cultured in the absence of GM-CSF and TNF-α they will become $CD68^+$ and show morphologic characteristics of macrophages after 5-6 days (M.O.Canning, personal communication).

In conclusion, DC are bone marrow derived and heterogeneous. Precursors of separate lineages of DC may circulate in the peripheral blood, one of these is a $CD14^+$ monocyte.

1.6.4 Relationship of DC to the monocyte-macrophage lineage

Considering the above, there is no consensus about the exact relationship of DC with monocytes and macrophages. There is evidence that a single cell precursor can give rise to both DC and macrophages[156,157]. The capacity of GM-CSF to induce DC-like cells from cord and adult blood, without outgrowth of the granulocyte series, might suggest a relatively late precursor in common with the monocyte-macrophage series. The above mentioned studies using the monocytic pool of the blood to produce DC[152,154,155], and recent reports[153,158] describing the possibility to differentiate DC from purified CD14-strongly positive cells, plea for a monocyte-type precursor for DC.
On the other hand, the lack of DC of strong phagocytotic activity, the constitutive rather than inducible MHC-class II expression, and their specialization as unique stimulators of the primary immune response are strong arguments for considering DC as a separate hemopoietic lineage[122,123].
Macrophages may help DC in exerting their function. It is described that macrophage factors like IL-10 enhance the response of cytotoxic T-cells to be stimulated by low numbers of DC[159]. However, alveolar macrophages have been reported to have a suppressive effect on DC function[160].

Macrophages also show a strong heterogeneity in phenotype and function[161]. There is no monoclonal antibody, both in human and in mice that recognizes all macrophages, and shows no cross-reactivity to other hematopoietic cells. F4/80 in the mouse has for some time been regarded as macrophage specific[162]. However, it was shown that there exists some cross reactivity with eosinophils[163]. Other studies showed that F4/80 was not present on specific subtypes of macrophages, including lysosome-

positive resident macrophages[164,165]. The lack of a common marker for all macrophage subpopulations is also suggestive for a functional heterogeneity of the cells. Among the many functions they perform are endocytosis of microorganisms and debris[166], cytotoxicity against microorganisms or tumor cells[167], and regulation of and participation in the inflammatory response[168]. They also perform a scavenger function, and are involved in the wound healing process[169,170], the regulation of the specific immune response[171,172] and the regulation of hemo- and lymphopoiesis[173,174]. This specialization in function likely results in the expression of specific proteins and other membrane constituents necessary for an adequate performance of function, and is probably also reflected in the variety of marker patterns. Not only the marker pattern differentiates between the various types of macrophages, but they also differ in their secretion products. In exerting their function, macrophages produce various products, several of which have been identified, but probably many are still not recognized. Among the important secretion products of macrophages are the cytokines IL-1, IL-6, IL-10, IFN-β, GM-CSF, and TNF-α. Radicals like NO and oxygen-radicals are mainly produced by scavenger and cytotoxic macrophages. Metabolites of the arachidonic acid metabolism are other important products of macrophages. The profile of the different secretion products in a certain type of macrophage is strongly dependent on its function[161]. Upon stimulation, macrophages can be induced to be activated or to differentiate into a more specialized state. This has implications for the profile of their secretion products[161].

In conclusion, macrophages and DC constitute heterogeneous populations both in marker pattern and in function. Their origin and lineages are still hazy, but are probably closely connected.

1.6.5 Dendritic cells and macrophages in IDDM

In an immunohistochemical examination of the autoimmune islet lesions in the BB-rat it was demonstrated that MHC class II^+ antigen presenting cells with a dendritic morphology were the first cells infiltrating the islets of Langerhans[175]. At later stages lymphocytes and scavenger macrophages (the latter strongly positive for acid phosphatase) were demonstrated around and in the islets. In the human situation it is impossible to study early cases of pre-diabetes histologically. Regarding the question of antigen-presentation, Bottazzo et al observed several years ago that β-cells in type 1 diabetics may express class II MHC antigens. The investigators therefore proposed that β-cells might become APC after aberrant induction of MHC class II on their membrane (by for instance a viral infection)[176]. Investigating this hypothesis, Wright[177] showed that induction of class II molecules on islets cells before transplantation by

incubation with TNF-α did not induce a rapid rejection, but in contrast prolonged the survival of islet allografts. Therefore, class II expression on the β-cell may rather be a defence mechanism of the β-cell against the immune reaction surrounding it.

The importance of MHC-class II^+ DC was recently illustrated by experiments of Clare-Salzler et al[178]: DC isolated from pancreatic lymph node of 8-20 week old NOD mice showed cluster formation with T cells from the same lymph node, three to twenty times higher than in control mice, and in other lymph nodes from the same animals. The pancreatic lymph node DC did not cluster with T cells from other lymph nodes of the same animal.

The capacity of DC to initiate an autoimmune response was demonstrated in another model for autoimmune disease: the thyroiditis model. Knight et al showed that DC pulsed with thyroglobulin were able to induce thyroiditis after cell transfer to normal non-autoimmune BALB/c mice[179].

The essential role of macrophages and macrophage-related cells in the development of diabetes has also been shown in *in vivo* experiments. Administration of silica, a compound toxic for macrophages prevented the development of insulitis and diabetes, both in the BB-rat[180,181] and in the NOD-mouse[182,183], when applied early in the disease. Late administration appeared to be ineffective[183]. Also a diet deficient in essential fatty acids, which almost completely eliminates macrophages, resulted in a considerable reduction in the diabetes incidence in BB rats from 85 to 30%[184]. Moreover, inhibiting the recruitment of inflammatory cells, including macrophages, with the help of 5C6, a monoclonal antibody to complement receptor 3, blocked the development of diabetes[185] in NOD mice in a transfer model.

Macrophages can be induced to synthesize large amounts of NO-radicals upon activation. High levels of NO impair the function of mitochondrial and other FeS-containing enzymes, and may also result in DNA-damage[186]. Islet β-cells are extremely sensitive to NO radicals[187]. In vitro experiments demonstrated that macrophage induced β-cell lysis did not occur in the absence of L-arginine, which is needed for NO-production[186]. Inhibitors of NO-synthetase are also effective in vitro to prevent β-cell death. Also nicotinamide, which protects the β-cell from depletion of its NAD-content[188] and probably acts as a radical-scavenger too, thereby diminishing DNA-damage[189], prevents β-cell death from NO-radicals. In vivo, nicotinamide was shown to be effective in preventing diabetes in NOD mice[188,190].

Another macrophage product, IL-1β, is described to have a prevailing inhibiting effect on glucose stimulated insulin secretion. Only with short exposure times, at low concentrations, and on resting β-cells IL-1β has a slight stimulating effect[191]. Finally, the macrophage product TNF-α, in combination with IFN-γ or IL-1, has been described to be able to induce or enhance β-cell death in vitro, not only in animal

models[192,193], but also in human islets[194].
With regard to macrophage function in the NOD mouse, it is an interesting observation that macrophages from NOD mice appear to have an aberrant macrophage secretion product profile: They are deficient in the production of IL-1[195] and TNF-α after stimulation with endotoxin in vitro[58]. Moreover, peritoneal macrophages from NOD mice, spontaneously and after stimulation, produce a significantly larger amount of 6-keto-prostaglandin F1α (PGF1α) and prostaglandin E2 (PGE2), than macrophages from control strains of mice (C57BL/6, BALB/c and DBA mice)[196]. Also the production of leukotrien C4 on stimulation with zymosan A was considerably higher in NOD mice than in the control C57BL/6 strain.

1.7 Aim and outline of the thesis

The aim of this thesis is to further investigate both on a histomorphological level as well as a functional level the role of DC and macrophage populations in IDDM, addressing the following questions:

a) Which DC and macrophage populations are normally present in the pancreas in and near the islets?
b) Which DC and macrophage populations play a role in the initiation of the β-cell directed autoimmune process in type 1 diabetes?
c) Which DC and macrophage populations are involved in β-cell destruction in the later phases of the β-cell directed autoimmune process in type 1 diabetes?
d) What is the functional activity of monocyte-derived DC in type 1 diabetes?

These questions are dealt with in the following chapters:

Chapter 2: *Immunohistochemistry of macrophages and DC in human pancreases*
An immunohistochemical study on the presence of DCs and macrophages in pancreases of normal fetuses and neonates (Q.a), as well as in the pancreas of an infant who died after rapid onset of IDDM (Q. b,c).

Chapter 3: *Immunohistochemistry of macrophages and DC in murine pancreases*
3.1 A sequential immunohistochemical study on the presence of various subclasses of DC and macrophages in and around the islets of NOD mice spontaneously developing IDDM (Q. a,b,c).
3.2 The effect of prophylactic insulin treatment on the pancreatic infiltration

of DC and macrophages in the NOD mouse (Q.b).

3.3 The effect of tolerance induction via intra-thymic injection of syngeneic islets on the pancreatic infiltration of macrophages in the NOD mouse (Q.c).

3.4 The effect of castration on the pancreatic infiltration of DC and macrophages in male NOD mice (Q. b,c).

Chapter 4: *Functional evaluation of DC in human IDDM*

A functional assessment of monocyte-derived DC (maturation, capability to form cellular clusters, stimulation in mixed lymphocyte reaction) of type 1 diabetic patients (Q.d)

REFERENCES

1. Tuomi T, Groop L, Zimmet P, Rowley M, Knowles W, Makay I. Antibodies to glutamic acid decarboxylase reveal latent autoimmune diabetes mellitus in adults with a non-insulin dependent onset of the disease. Diabetes 42:359-362,1993
2. Groop L, Bottazzo GF, Doniach D. Secondary failure treatment with oral antidiabetic agents in non-insulin dependent diabetes. Diabetes Care 9:129-132,1986
3. Temple RC, Carrington CA, Luzio SD, Owens DR, Schneider AE, Sobey WJ, Hales CN. Insulin deficiency in non-insulin-dependent diabetes. Lancet 1:293-295,1989
4. Laporte RE, Tajima N, Åkerblom HK, Berlin N, Brosseau J, Christy AL, Fishbein HA, Green A, Hamman R, Harris M, King H, Laron Z, Neil A. Geographic patterns in the risk of insulin-dependent diabetes mellitus: the importance of registries. Diabetes Care 8 (suppl 1):101-107,1985
5. Barnett AH, Eff C, Leslie RDG, Dijke DA. Diabetes in identical twins: A study of 200 pairs. Diabetologia 20:87093,1981
6. Olmos P, A'Hern R, Heaton DA, Risley D, Pyke DA. The significance of the concordance rate of type-1 (insulin dependent) diabetes in identical twins. Diabetologia 31:747-750,1988
7. Faustman D, Li XP, Lin HY, Fu YE, Eisenbarth G, Avruch J, Guo J. Linkage of faulty major histocompatibility complex class I to autoimmune diabetes. Science 254:1756-1761,1991
8. Wolf E, Spencer KM, Cudworth AG. The genetic susceptibility to type I (insulin-dependent) diabetes: analysis of the HLA-DR association. Diabetologia 24:224-230,1983
9. Rotter JI, Anderson CE, Rubin R, Congleton JE, Terasaki PI, Rimoin DL. HLA genotypic study of insulin-dependent diabetes; excess of DR3/DR4 heterozygotes allows rejection of the recessive hypothesis. Diabetes 32:169-174,1983
10. Svejgaard A, Platz P, Ryder LP. HLA and disease 1982 - a survey. Immunol Rev 70:193-218,1983
11. Todd JA, Acha-Orbea H, Bell JI et al. A molecular basis for MHC class II associated autoimmunity. Science 240:1003-1009,1988
12. Todd JA, Bell JI, McDevitt HO. HLA-DQ beta gene contributes to susceptibility to insulin-dependent diabetes mellitus. Nature 329:599,1987
13. Kahlil I, d'Auriol L, Gobet M, Morin L, Lepage V, Deschamps I, Park MS, Degos L, Galbert F, Hors J. A combination of HLA-DOβ Asp57-negative and HLA-DQα Arg52 confers susceptibility to insulin-dependent diabetes mellitus. J Clin Invest 85:1315-1319,1990
14. Bain SC, Prins JB, Hearne CM, Rodriques NR, Rowe BR, Pritchard LE, Ritchie RJ et al. Insulin gene region-encoded susceptibility to type I diabetes is not restricted to HLA-DR4-positive individuals. Nature Genet 2:212-215,1992
15. Heaton DA, Millward BA, Gray IP, Yun Y, Hales CN, Pyke DA, Leslie RDG. Increased proinsulin levels as an early indicator of beta-cell dysfunction in non-diabetic twins of type I (insulin-dependent) diabetic patients. Diabetologia 31:182-184,1988
16. Hartling SG, Lindgren F, Dahgvist G, Persson B, Binder C. Elevated proinsulin in healthy siblings of IDDM patients independent of HLA identify. Diabetes 38:1271-1274,1989
17. Gepts W. Pathologic anatomy of the pancreas in juvenile diabetes mellitus. Diabetes 14:619-633,1965

18. Foulis AK, Liddle CN, Farquharson MA, Richmond JA, Weir RS. The histopathology of the pancreas in type 1 (insulin-dependent) diabetes mellitus: a 25-year review of deaths in patients under 20 years of age in the United Kingdom. Diabetologia 29:267-274, 1986
19. Bottazzo GF, Dean MRC, McNally JM, MacKay EH, Swift PGF, Gamble DR. In situ characterization of autoimmune phenomena and expression of HLA molecules in the pancreas in diabetic insulitis. New Eng J Med 313:353-360,1985
20. Bottazzo GF, Florin-Christensen A, Doniah D. Islet cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies. Lancet 2:1279-1283,1974
21. Lendrum R, Walker JG, Gamble DR. Islet cell antibodies in juvenile diabetes mellitus of recent onset. Lancet 1:880-882,1975
22. Lendrum R, Walker G, Cudworth A, Theophanides C, Pyke DA, Bloom A, Gamble DR. Islet cell antibodies in diabetes mellitus. Lancet 2:1273-1276,1976
23. Landin-Olsson M, Karlsson A, Dahlquist G, Blom L, Lernmark Å, Sundkvist G. Islet cell and other specific autoantibodies in all children developing type 1 diabetes mellitus in Sweden during one year and in matched control children. Diabetologia 32:387-395,1989
24. Drell DW, Notkins AL. Multiple immunological abnormalities in patients with type I (insulin-dependent) diabetes mellitus. Diabetologia 30:132-134,1987
25. Ziegler AG, Herskowitz RD, Jackson RA, Soeldner JS, Eisenbarth GS. Predicting type I diabetes. Diabetes Care 13:762-775,1990
26. Ko IY, Ihm SH, Yoon JW. Studies on autoimmunity for initiation of beta-cell destruction. VIII. Pancreatic beta-cell dependent autoantibody to a 38 kilodalton protein precedes the clinical onset of diabetes in BB rats. Diabetologia 34:548-554,1991
27. Martin JM, Trink B, Daneman D, Dosch HM, Robinson B. Milk proteins in the etiology of insulin-dependent diabetes mellitus (IDDM). Ann Med 23:447-452,1991
28. Cataño L, Russo E, Zhou L, Lipes MA, Eisenbarth GS. Identification and cloning of a granule autoantigen (carboxypeptidase-H) associated with type 1 diabetes. J Clin Endocrinol Metab 73:1197-1201,1991
29. Baekkeskov S, Aanstoot H-J, Christgau S, Reetz A, Solimena M, Cascalho M, Folli F, Richter-Olesen H, de Camilli P. Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature 347:151-156,1990
30. Karlsen AE, Hagopian WA, Grubin CE, Dube S, Disteche CM, Adler DA, Bårmeier H, Mathewes S, Grant FJ, Foster D, Lernmark Å. Cloning and primary structure of a human islet isoform of glutamic acid decarboxylase from chromosome 10. Proc Natl Acad Sci USA 88:8337-8341,1991
31. Ziegler AG, Ziegler R, Vardi P, Jackson RA, Soldner JS, Eisenbarth GS. Life-table analysis of progression to diabetes of anti-insulin autoantibody-positive relatives of individuals with type 1 diabetes. Diabetes 38:1320-1325,1989
32. Yale J, Grose M, Marliss EB. Time course of the lymphopenia in BB rats. Diabetes 34:954-959,1985
33. Greiner DL, Handler ES, Nakono K, Mordes JP, Rossini AA. Absence of the RT-6 T cell subset in diabetes prone BB/W rats. J Immunol 136:148-151,1986
34. Makino S, Kunimoto K, Muraoka Y, Mizushima Y, Katagiri K, Tochino Y. Breeding of a non-obese diabetic strain of mice. Exp Anim 29:1-3,1980
35. Pozzilli P, Signore A, Williams AJK, Beales P. NOD mouse colonies around the world-recent facts and figures. Immunology Today 14:193-196,1993
36. Coleman DL, Kuvaza JE, Leiter EH. Effect of diet on incidence of diabetes in non-obese diabetic mice. Diabetes 39: 432-436,1990
37. Williams A, Lampeter EF, Signore A, Beales PE, Mansfiled K, Gale EAM, Ward T, Pozzilli P. Increase in environmental temperature reduces diabetes incidence in the non-obese-diabetic mouse. Diabetologia 32:557A,1989
38. Oldstone MBA. Prevention of type I diabetes in nonobese diabetic mice by virus infection. Science 239:500-502,1988
39. Wilberz S, Parke HJ, Dagnaes-Hansen F, Herberg L. Persistent MHV infection reduces the incidence of diabetes mellitus in non-obese diabetic nice. Diabetologia 34:2-5,1991
40. Makino S, Kunimoto K, Muraoka Y, Katagiri K. Effect of castration on the appearance of diabetes in NOD mouse. Exp Anim 30:137-140,1981
41. Fitzpatrick F, Lepault F, Homo-Delarche F, Bach JF, Dardenne M. Influence of castration, alone or combined with thymectomy, on the development of diabetes in the nonobese diabetic mouse. Endocrinology 129:1382-1390,1991
42. Shieh D-C, Cornelius J, Winter W, et al. Insulin-dependent diabetes in the NOD mouse model. I. Detection and characterization of autoantibody bound to the surface of pancreatic beta-cells prior to the development of the insulitis lesion in prediabetic NOD mice. Autoimmunity 15:123-135,1993
43. Tisch R, Yang X-D, Singer S, et al. Immune response to glutamic acid decarboxylase correlates with insulitis in non-obese diabetic mice. Nature 366:72-75,1993

44. Serreze DV, Leiter EH, Kuff EL, et al. Molecular mimicry between insulin and retroviral antigen p73. Development of cross-reactive autoantibodies in sera of NOD and C57BL/KsJ-db/db mice. Diabetes 37:351-358,1988
45. Lehuen A, Altman J, Bach JF, Carnaud C. Natural thymocytotoxic autoantibodies in non-obese diabetic (NOD) mice: characterization and fine specificity. Clin Exp Immunol 81:406-411,1990
46. Makino S, Hayashi Y,Muraoka Y, Tochino Y. Establishment of non-obese diabetic (NOD) mouse. Current topics in Clinical and Experimental aspects of Diabetes Mellitus. Eds N Sakamoto, HK Min, S Baba. Amsterdam, Elsevier International Congress Series 717:29-36,1986
47. Many MC, Denef JF. Two-step development of Iodine-induced thyroiditis in goitrous autoimmune prone non obese diabetic mice. J Endocrin Invest 15:26,1992
48. Gosh S, Palmer SM, Rodriques NR, Cordell HJ, Hearne CM, Cornall RJ, Prins J-B, McShane P, Lathrop GM, Peterson LB, Wicker LS, Todd JA. Polygenic control of autoimmune diabetes in nonobese diabetic mice. Nature Genetics, 4:404-409,1993
49. Prins J-B, Todd JA, Rodriques NR, Gosh S, Hogarth PM, Wicker LS, Gaffney E, Podolin PL, Fisher PA, Sirotina A, Peterson LB. Linkage on chromosome 3 of autoimmune diabetes and defective Fc receptor for IgG in NOD mice. Science 260:695-698,1993
50. Garchon H-J, Luan J-J, Eloy L, Bédossa P, Bach J-F. Genetic analysis of immune dysfunction in non-obese diabetic (NOD) mice: mapping of a susceptibility locus close to the Bcl-2 gene correlates with increased resistance of NOD T cells to apoptosis induction. Eur J Immunol 24:380-384,1994.
51. Mori Y, Suko M, Okudaira H, Matsuba I, Tsuruoka A, Sasaki A, Yokoyama H, Tanase T, Shida T, Nishimura M, et al. Preventive effects of cyclosporine on diabetes in NOD mice. Diabetologia 29:244-247,1986
52. Miyagawa J, Yamamoto K, Hanafuasa T, Itoh N, Nakagawa C, Otsuka A, Katsura H, Yamagata K, Miyazaki A, Kono N, et al. Preventive effect of a new immunosuppressant FK-506 on insulitis and diabetes in non-obese diabetic mice. Diabetologia 33:503-505,1990
53. Lipton R, Laporte RE, Becker DJ, Dorman JS, Orchard TJ, Atchison J, Drash AL. Cyclosporin therapy for prevention and cure of IDDM. Epidemiological perspective of benefits and risks. Diabetes care 13:776-784,1990
54. Serreze DV, Hamaguchi K, Leiter EH. Immunostimulation circumvents diabetes in NOD/Lt mice. J Autoimmunity 2:759-776,1989
55. Rapoport MJ, Jamarillo A, Zipris D, Lazarus AH, Serreze DV, Leiter EH, Cyopick P, Danska JS, Delovitch TL. IL-4 reverses T-cell proliferative unresponsiveness and prevents the onset of diabetes in NOD mice. J exp Med 178:87-99,1993
56. Pennline KJ, Roque-Gaffney E, Monahan M. Recombinant human IL-10 (rHu IL-10) prevents the onset of diabetes in the non-obese diabetic (NOD) mouse. Clin Immunol Immunopathol 71:169-175,1994
57. Wilson CA, Jacobs C, Baker P, Baskin DG, Dower S, Lernmark Å, Toivola B, Vertrees S, Wilson D. IL-1β modulation of spontaneous autoimmune diabetes and thyroiditis in the BB rat. J Immunol 144:3784-3788,1990
58. Jacob CO, Asiso S, Michie SA, McDevitt HO, Acha-Orbea H. Prevention of diabetes in non-obese diabetic mice by tumor necrosis factor (TNF): similarities between TNF-α and interleukin-1. Proc Natl Acad Sci USA 87:968-972,1990
59. Satoh J, Seino H, Abo T, Tanaka S-I, Shintani S, Ohta S, Sawai T, Nobunga T, Oteki T, Kumagai K, Toyota T. Recombinant human tumor necrosis factor-α suppresses autoimmune diabetes in nonobese diabetic mice. J Clin Invest 84:1345-1348,1989
60. Sadelain MWJ, Qin H-Y Lauzon J, Singh B. Prevention of type I diabetes in NOD mice by adjuvant immunotherapy. Diabetes 39:583-589,1990
61. Shehadeh N, Calcinaro F, Bradley BJ, Bruchlim I, Vardi P, Lafferty KJ. Effect of adjuvant therapy on development of diabetes in mouse and man. Lancet 343:706-707,1994
62. Gotfredsen CF, Buschard K, Frandsen EK. Reduction of diabetes incidence of BB Wistar rats by early prophylactic insulin treatment of diabetes-prone animals. Diabetologia 28:933-935; 1985
63. Atkinson MA, Maclaren NK, Luchetta R. Insulitis and diabetes in NOD mice reduced by prophylactic insulin therapy. Diabetes 39: 933-937,1990.
64. Keller RJ, Eisenbarth GS, Jackson RA. Insulin prophylaxis in individuals at high risk of type I diabetes. Lancet 341: 927-928,1993
65. Miyazaki A, Hanafusa T, Yamada K, Miyagawa J, Fujino-Kurihara H, Nakajima H, Nonaka K, Tarui S. Predominance of T-lymphocytes in pancreatic islets and spleen of pre-diabetic non-obese diabetic (NOD) mice: a longitudinal study. Clin Exp Immunol 60:622-630,1985
66. Like AA, Kislauskis E, Williams RM, Rossini AA. Neonatal thymectomy prevents spontaneous diabetes mellitus in BB/W rat. Science 216:644-646,1982
67. Ogawa M, Marayuama T, Hasegawa T et al. The inhibitory effect of neonatal thymectomy on the incidence of insulitis in non-obese (NOD) mice. Biomed Res 6:103-105,1985

68. Ikehara S, Ohtsuki H, Good RA et al. Prevention of type 1 diabetes in nonobese diabetic (NOD) mice by allogeneic bone marrow transplantation. Proc Natl Acad Sci USA 82:7743-7747,1985
69. Prochazka M, Gaskins HR, Schultz LD, Leiter EH. The NOD-*scid* mouse: a model for spontaneous thymomagenesis associated with immunodeficiency. Proc Natl Acad Sci USA 89:3290-3294,1992
70. Bendelac A, Carnaud C, Boitard C Bach JF. Syngeneic transfer of autoimmune diabetic NOD mice to healthy neonates: requirement for both L3T4$^+$ and Lyt-2$^+$ T cells. J Exp Med 166:823-832,1987
71. Miller BJ, Appel MC, O'Neill JJ, Wicker LS. Both the Lyt-2$^+$ and L3T4$^+$ T cells subsets are required for the transfer of diabetes in nonobese diabetic mice. J Immunol 140:52-58,1988
72. Haskins K, McDuffie M. Acceleration of diabetes in young NOD mice with a CD4$^+$ islet-specific T cell clone. Science 249:1433-1436,1990
73. Christianson SW, Shultz LD, Leiter EH. Adoptive transfer of diabetes into immunodeficient NOD-*scid/scid* mice. Relative contributions of CD4$^+$ and CD8$^+$ T-cells from diabetic versus prediabetic NOD.NON-Thy-1^a donors. Diabetes 42:44-55,1993
74. Mosmann TR, Coffman RL. TH1 and TH2 cells: Different patterns of lymphokine secretion lead to different functional properties. Annu Rev Immunol 7:145-173,1989
75. Romagnani S. Human TH1 and TH2 subsets: regulation of differentiation and role in protection and immunopathology. Int Arch Allergy Immunol 98:279-285,1989
76. Hsieh C-S, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM. Development of TH1 CD4$^+$ T-cells through IL-12 produced by Listeria-induced macrophages. Science 260:496-497,1993
77. Scott P. IL-12: initiation cytokine for cell-mediated immunity. Science 260:496-497,1993
78. Moore KV, O'Garra A, de Waal Malefyt R, Vieira P, Mosmann TR. Interleukin 10. Annu Rev Immunol 11:165-190,1993
79. D'Andrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M, Trinchieri G. Interleukin 10 (IL-10) inhibits human lymphocyte interferon-γ production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. J Exp Med 178:1041-1048, 1993
80. Powrie F, Menon S, Coffman RL. Interleukin-4 and interleukin-10 synergize to inhibit cell-mediated immunity in vivo. Eur J Immunol 23:2223-2229,1993
81. Quill H, Gaur A, Phipps RP. Prostaglandin E2-dependent induction of granulocyte-macrophage colony-stimulating factor secretion by cloned murine helper T cells. J Immunol 142:813-818,1989
82. Betz M, Fox BS. Prostaglandin E2 inhibits production of Th1 lymphokines but not of Th2 lymphokines. J Immunol 146:108-113,1991
83. Munoz E, Zubiaga AM, Merrow M, Sauter NP, Huber BT. Cholera toxin discriminates between T helper 1 and 2 cells in T cell receptor-mediated activation: role of cAMP in T cell proliferation. J Exp Med 172:95-103,1990
84. Johnson HM, Torres BA. Leukotrienes: positive signals for regulation of γ-interferon production. J Immunol 132:413-416,1984
85. Sarvetnick N, Fox HS. Interferon-γ and the sexual dimorphism of autoimmunity. Mol Biol Med 7:323-331,1990
86. Campbell IL, Kay TWH, Oxbrow L, Harrison LC. Essential role for interferon-γ and interleukin-6 in autoimmune insulin-dependent diabetes in NOD/Wehi mice. J Clin Invest 87:739-742,1991
87. Shehadeh N, LaRosa F, Lafferty KJ. Altered cytokine activity in adjuvant inhibition of autoimmune diabetes. J Autoimmunity 6:291-300,1993
88. Rabinovitch A, Sorensen O, Suarez-Pinzon WL, Power RF, Rajotte RV, Bleackley RC. Analysis of cytokine mRNA expression in syngeneic islet grafts of NOD mice: interleukin-2 and interferon-γ mRNA expression correlate with graft rejection and interleukin-10 with graft survival. Diabetologia 37:833-837,1994
89. Townsend A, Bodmer H. Antigen recognition by class I-restricted T lymphocytes. Annu Rev Immunol 7:601-624,1989
90. Rudensky AY, Preston-Hulburt P, Hong SC, Barlow A, Janeway CA. Sequence analysis of peptide bound to MHC class II molecules. Nature 353:622-627,1991
91. Roche PA, Cresswell P. Invariant chain association with HLA-DR molecules inhibits immunogenic peptide binding. Nature 345:615-618,1990
92. Teyton L, O'Sullivan D, Dickson PW, Lotteau V, Sette A, Fink P, Peterson PA. Invariant chain distinguishes between the exogenous antigen presentation pathways. Nature 348:39-44,1990
93. Schumacher TNM, DeBruijn MLH, Vernie LN, Kast WM, Melief CJ, Neefjes JJ, Ploegh HL. Peptide selection by MHC class I molecules. Nature 350:703-706,1991
94. Monaco JJ, McDevitt HO. H-2 linked low-molecular weight polypeptide antigens assemble into an unusual macromolecular complex. Nature 309:797-799,1984
95. Driscoll J, Goldberg AL. The proteasome (multicatalytic protease) is a component of the 1500-kDa proteolytic complex which degrades ubiquitin-conjugated proteins. J Biol Chem 265:4789-4792,1990

96. Spies T, DeMars R. Restored expression of major histocompatibility class I molecules by gene transfer of a putative peptide transporter. Nature 351:323-324,1991
97. Attaya M, Jameson S, Martinez CK, Hermel E, Aldrich C, Forman J, Lindahl KF, Bevan MJ, Monaco JJ. Ham-2 corrects the class I antigen-processing defect in RMA-S cells. Nature 355:647-649,1992
98. Falk K, Rotschke O, Stevanovic S, Jung G, Rammensee HG. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. Nature 351:290-296,1991
99. Bierer BE, Sleckman BP, Ratnofsky SE, Burakoff SJA. The biologic roles of CD2, CD4, and CD8 in T-cell activation. Annu Rev Immunol 7:579-599,1989
100. Norton SD, Zuckerman L, Uhrdahl KB, Shefner R, Miller R, Jenkins MK. The CD28 ligand B7 enhances IL-2 production by providing a co-stimulatory signal to T-cells. J Immunol 149:1556-1561,1992
101. June CH, Bluestone JA, Nadler LM, Thompson CB. The B7 and CD28 receptor families. Immunology Today 15:321-331,1994
102. Mueller DL, Jenkins MK, Schwartz RH. Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T-cell antigen receptor occupancy.Ann Rev Immunol 7:445-480,1989
103. Kishimoto TK, Larson RS, Corbi AL, Dustin ML, Staunton DE, Springer TA. The leukocyte integrins. Adv Immunol 46:149-182,1989
104. Inaba K, Steinman RM. Resting and sensitized T Lymphocytes exhibit distinct stimulatory (antigen presenting cell) requirements for growth and lymphokine release. J Exp Med 160:1717-1735,1984
105. Van Voorhis WC, Valinsky J, Hoffman E, Luban J, Hair LS, Steinman RM. Relative efficiency of human monocytes and dendritic cells as accessory cells for T cell replication. J Exp Med 158:174-191,1983
106. Linsley PS, Clark EA, Ledbetter JA. T-cell antigen CD28 mediates adhesion with B ells by interacting with activation antigen B7/BB1. Proc Natl Acad Sci USA 87:5031-5035,1990
107. Flechner ER, Freudenthal PS, Kaplan G, Steinman RM. Antigen specific T lymphocytes efficiently cluster with dendritic cells in the human primary mixed leucocyte reaction. Cell Immunol 111:183-195,1988
108. Austyn JM, Weinstein DE, Steinman RM. Clustering with dendritic cells precedes and is essential for T-cell proliferation in a mitogenesis model. Immunology 63:691-696,1988
109. Inaba K, Steinman RM. Accessory cell-T lymphocyte interactions. Antigen dependent and independent clustering. J Exp Med 163:247-261,1986
110. Austyn JM, Smith KG, Morris PJ. T cell activation by anti-CD3 antibodies: function of Fc receptors on B cell blasts, but not resting B cells, and CD18 on the responding T cells. Eur J Immunol 17:1329-1335,1987
111. Van Voorhis WC, Hair LS, Steinman RM, Kaplan G. Human dendritic cells. Enrichment and characterization from peripheral blood. J Exp Med 155:1172-1187,1982
112. Romani N, Schuler G. The immunologic properties of epidermal Langerhans cells as part of the dendritic cell system. Springer Semin Immunopathol 13:265-279,1992
113. Puré E, Inaba K, Crowley MT, Tardelli L, Witmer-Pack MD, Ruberti G, Fathman G, Steinman RM. Antigen processing by epidermal Langerhans cell correlates with the level of biosynthesis of major histocompatibility complex class II molecules and expression of invariant chain. J Exp Med 172:1459-1469,1990
114. Harding CV, Roof RW, Unanue ER. Turnover of Ia-peptide complexes is facilitated in viable antigen-presenting cells: Biosynthesis turnover of Ia vs peptide exchange. Proc Natl Acad Sci USA 86:4230-4234,1990
115. Nussenzweig MC, Steinman RM, Witmer MD, Gutchinov B. A monoclonal antibody specific for mouse dendritic cells. Proc Natl Acad Sci USA 79:161-165,1982
116. Kraal G, Breel M, Janse M, Bruin G. Langerhans' cells, veiled cells and interdigitating cells in the mouse recognized by a monoclonal antibody. J Exp Med 163:981-997,1986
117. Metlay JP, Witmer-Pack MD, Agger R, Crowley MT, Lawless D, Steinman RM. The distinct leukocyte integrins of mouse spleen dendritic cells as identified with new hamster monoclonal antibodies. J Exp Med 171:1753-1771,1990
118. Agger R, Witmer-Pack MD, Romani N, Stossel H, Swiggard WJ, Metlay JP, Storozynski E, Freimuth P, Steinman RM. Two populations of splenic dendritic cells detected with M342, a new monoclonal to an intracellular antigen of interdigitating dendritic cells and some B lymphocytes. J Leuk Biol 52:34-42,1992
119. Davis AL, McKenzie JL, Hart DNJ. HLA-DR positive leucocyte subpopulations in human skin include dendritic cells, macrophages, and CD7 negative T cells. Immunology 65:573-581,1988

120. Prickett TCR, McKenzie JL, Hart DNJ. Characterization of interstitial dendritic cells in human liver. Transplantation 46:754-761,1988
121. Freudenthal PS, Steinman RM. The distinct surface of human blood dendritic cells, as observed after an improved isolation method. Proc Natl Acad Sci 87:7698-7702,1990
122. Austyn JM. Lymphoid dendritic cells. Immunology 62:161-170,1987
123. Steinman RM. The dendritic cell system and its role in immunogenicity. Ann Rev Immunol 9:271-196,1991
124. Prickett TCR, McKenzie JL, Hart DNJ, Adhesion molecules of human tonsil dendritic cells. Transplantation 53:483-490,1992
125. Hart DNJ, Prickett TCR. Intercellular adhesion molecule-2 (ICAM-2) expression on human dendritic cells. Cell Immunol 148:447-454,1993
126. Inaba K, Steinman RM. Monoclonal antibodies to LFA-1 and to CD4 inhibit the mixed leucocyte reaction after the antigen dependent clustering of dendritic cells and T lymphocytes. J Exp Med 165:1403-1417,1987
127. Hart DNJ, Starling GC, Calder VL, Fernando NS. B7/BB1 is a leucocyte differentiation antigen on human dendritic cells induced by activation. Immunol 79:616-620,1993
128. Hart DNJ, Fabre JW. Demonstration and characterization of Ia-positive dendritic cells in the interstitial connective tissues of rat hart and other tissues but not brain. J Exp Med 153:347-361,1981
129. Hart DNJ, Fabre JW. MHC antigens in rat kidney, ureter and bladder: localization and demonstration of Ia positive dendritic cells. Transplantation 31:318-325,1981
130. Daar AS, Fuggle SV, Hart DNJ, Dalchau R, Abdulaziz Z, Fabre JW, Ting A, Morris PJ. Demonstration and phenotypic characterization of HLA-DR positive interstitial dendritic cells widely distributed in human connective tissues. Transplant Proc 15:313-315,1983
131. Darden AG, Clarke Forbes RD, Darden PM, Guttman RD. The effect of genetics and age on expression of MHC class II and CD4 antigens on rat cardiac interstitial dendritic cells. Cell Immunol 126:322-330,1990
132. Larsen CP, Morris PJ, Austyn JM. Migration of dendritic leukocytes from cardiac allografts into host spleens: a novel route for initiation of rejection. J Exp Med 171:307-314,1990
133. Romani N, Lenz A, Glassel H, Stossel H, Stanzl U, Majdic O, Fritsch P, Schuler G. Cultured human Langerhans' cells resemble lymphoid dendritic cells in phenotype and function. J Invest Dermatol 93:600-609,1989
134. Teunissen MBM, Wormmeester MT, Krieg SR, Peters PJ, Vogels IM, Kapsenberg ML, Bos JD. Human epidermal Langerhans cells undergo profound morphologic and phenotypical changes during *in vitro* culture. J Invest Dermatol 94:166-173,1990
135. Witmer MD, Steinman RM. The anatomy of peripheral lymphoid tissues with emphasis on accessory cells: light microscopic immunocytochemical studies of mouse spleen, lymph node and Peyer's patch. Am J Anat 170:465-481,1984
136. Budjoso R, Hopkins J, Dutia BM, Young P, McDonell I. Characterization of sheep afferent lymph dendritic cells and their role in antigen carriage. J Exp Med 170:1285-1302,1989
137. Spry CJF, Pflug AJ, Janossy G, Humphrey JH. Large mononuclear veiled cells with Ia-like membrane antigens in human afferent lymph. Clin Exp Immunol 39:750-755,1980
138. Pugh CW, McPherson GG, Steer HW. Characterization of non-lymphoid cells derived from rat peripheral lymph. J Exp Med 157:1758-1779,1983
139. Hart DNJ, Calder VL. Human dendritic cells: Function and cytokine production. In: The handbook of Immunopharmacology. Immunopharmacology of macrophages and other antigen presenting cells. Eds: CAFM Bruijnzeel-Koomen and ECN Hoefsmit.Academic press Limited, London, 1994
140. Wood GS, Turner RR, Shiurba RA, Eng L, Warnke RA. Human dendritic cells and macrophages: *in situ* immunophenotypic definition of subsets that exhibit specific morphologic and microenvironmental characteristics. Am J Pathol 119:73-82,1985
141. Hart DNJ, McKenzie JL. Isolation and characterization of human tonsil dendritic cells. J Exp Med 168:157-170,1988
142. Steinman RM, Witmer MD, Kaplan G, Cohn ZA. Purification of spleen dendritic cells, new surface markers, and maintenance *in vitro*. J Exp Med 149:1-16,1979
143. Klinkert WEF, Labadie JH, Bowers WE. Accessory and stimulating properties of dendritic cells and macrophages isolated from various rat tissues. J Exp Med 156:1-19,1982
144. Barclay AN, Mayrhofer CJ. Bone-marrow origin of Ia positive cells in the medulla of rat thymus. J Exp Med 153:1666-1671,1981
145. Fowlkes BJ, Pardoll DM. Molecular and cellular events of T-cell development. Adv Immunol 44:207-264,1989

146. Inaba K, Witmer-Pack MD, Inaba M, Muramatsu S, Steinman RM. The function of Ia$^+$ dendritic cells and Ia$^-$ dendritic cell precursors in thymocyte mitogenesis to lectin and lectin plus interleukin 1. J Exp Med 167:149-162,1988
147. Volc-Platzer B, Stingl G, Wolff K, Hinterberg W, Schredl W. Cytogenetic identification of allogeneic epidermal Langerhans cells in a bone marrow graft recipient. N Eng J Med 310:1123-1124,1984
148. Knight SC, Farrant J, Bryant A, Edwards S, Burman S, Lever A, Clarke J, Webster AD. Non-adherent, low-density cells from human peripheral blood contain dendritic cells and monocytes, both with veiled morphology. Immunology 57:595-603,1986
149. Inaba K, Steinman RM, Witmer-Pack MD, Aya H, Inaba M, Sudo T, Wolpe S, Schuler G. Identification of proliferating dendritic cell precursors in mouse blood. J Exp Med 175:1157-1168,1992
150. Santiago-Schwartz F, Belilos E, Diamond B, Carsons SE. TNF in combination with GM-CSF enhances the differentiation of neonatal cord blood stem cells into dendritic cells and macrophages. J Leukoc Biol 52:274-281,1992
151. Steinbach F, Krause B, Thiele B. Monocyte derived cells represent phenotype and functional activities of Langerhans cells/dendritic cells. Abstract presented at the 3rd international symposium on dendritic cells in fundamental and clinical immunology, 1994, Annecy, France
152. Xu H, Kraemer M, Peters JH. Human monocytes treated with a combination of IL-4, GM-CSF, and IFN-gamma exhibit phenotype and T-cell stimulating potency comparable to blood dendritic cells. Abstract presented at the 3rd international symposium on dendritic cells and macrophages in fundamental and clinical immunology, 1994, Annecy, France
153. Caux C, Massacrier C, Vandervliet B, Dubois B, Dezutter-Dambuyant C, Schmitt D, Banchereau J, Schering-Plough. Characterization of human CD34$^+$ derived dendritic/langerhans cells (DLC). Abstract presented at the 3rd international symposium on dendritic cells in fundamental and clinical immunology 1994, Annecy, France
154. Kabel PJ, de Haan-Meulman M, Voorbij HAM, Kleingeld M, Knol EF, Drexhage HA. Accessory cells with a morphology and marker pattern of dendritic cells can be obtained from elutriator-purified blood monocyte fractions. An enhancing effect of metrizamide in this differentiation. Immunobiology 179:395-411,1989
155. Mooij P, Simons PJ, de Haan-Meulman M, de Wit HJ, Drexhage HA. Effect of thyroid hormones and other iodinated compounds on the transition of monocytes into veiled/dendritic cells: role of granulocyte-macrophage colony-stimulating factor, tumour-necrosis factor-α and interleukin-6. J Endocrin 140:503-512,1994
156. Goordyal P, Isaacson PG. Immunocytochemical characterization of monocyte colonies of human bone marrow: a clue to the origin of Langerhans cells and interdigitating reticulum cells. J Pathol 146:189-195,1985
157. Reid CDL, Stackpoole A, Meager A, Tikerpea J. Interactions of tumour necrosis factor with granulocyte-macrophage colony-stimulating factor and other cytokines in the regulation of dendritic cell growth *in vitro* from early bioptent CD34$^+$ progenitors in human bone marrow. J Immunol 149:2681-2688,1992
158. Schuler G. Production and properties of large numbers of dendritic cells from human blood. Lecture held at the 3rd international symposium on dendritic cells in fundamental and clinical immunology, 1994, Annecy, France
159. Naito K, Inaba K, Hirayama Y, Inaba-Miyama M, Sudo T, Muramatsu S. Macrophage factors which enhance the mixed leucocyte reaction initiated by dendritic cells. J Immunol 142:1834-1839,1989
160. Holt PG, Schon-Hegrad MA, Oliver J. MHC class II antigen-bearing dendritic cells in pulmonary tissues of the rat: regulation of antigen presentation activity by endogenous macrophage populations. J Exp Med 167:262-274,1988
161. Leenen PJM, Campbell PA. Heterogeneity of mononuclear phagocytes. An interpretive review. In: Blood Cell Biochemistry, volume 5: macrophages and related cells. Ed.: MA Horton. Plenum press New York, 1993
162. Austyn JM, Gordon S. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. Eur J Immunol 11:805-815,1981
163. Mc Garry MP, Stewart CC. Murine eosinophil granulocytes bind the murine macrophage-monocyte specific monoclonal antibody F4/80. J Leukoc Biol 50:471-478,1991
164. Kraal G, Rep M, Janse M. Macrophages in T and B cell compartments and other tissue macrophages recognized by monoclonal antibody MOMA-2. Scand J Immunol 26:653-661,1987
165. Keshav S, Chung L-P, Milon G, Gordon S. Lysosome is an inducible marker of macrophage activation in murine tissues as demonstrated by *in situ* hybridization. J Exp Med 174:1049-1058,1991

166. Griffin FM, Griffin JA, Leider JE, Silverstein SC. Studies on the mechanism of phagocytosis. I. Requirements for circumferential attachment of particle-bound ligands to specific receptors on the macrophage plasma membrane. J Exp Med 142:1263-1282,1975
167. Campbell PA, Canono BP, Cook JL. Mouse macrophages stimulated by recombinant gamma interferon to kill tumor cells are not bactericidal for the facultative intracellular bacterium *Listeria monocytogenes*. Infect Immunol 56:1371-1375,1988
168. Rosen H, Gordon S. The role of the type 3 complement receptor in the induced recruitment of myelomonocytic cells to inflammatory sites in the mouse. Am J Respir Mol Biol 3:3-10,1990
169. Leibovich SJ, Polverini PJ, Shephard HM, Wiseman DM, Shively V, Nuseir N. Macrophage-induced angiogenesis is mediated by tumour necrosis factor-α. Nature 329:630-632,1987
170. Pierce GF, Mustoe TA, Lingelbach J, Masakowski VR, Gramates P, Deuel TF. Transforming growth factor β reverses the glucocorticoid-induced wound-healing deficit in rats: possible regulation in macrophages by platelet-derived growth factor. Proc Natl Acad Sci USA 86:2229-2233,1989
171. Weaver CT, Unanue ER. The costimulatory function of antigen-presenting cells. Immunol Today 11:49-55,1990
172. Tomioka H, Saito H. Characterization of immunosuppressive functions of peritoneal macrophages induced with various agents. J Leukoc Biol 51:24-31,1992
173. Greenberger JS. The hematopoietic microenvironment. Crit Rev Oncol Hematol 11:65-84,1991
174. Van Ewijk W. T-cell differentiation is influenced by thymic microenvironments. Ann Rev Immunol 9:591-615,1991
175. Voorbij HAM, Jeucken PHM, Kabel PJ, de Haan M, Drexhage HA. Dendritic cells and scavenger macrophages in pancreatic islets of prediabetic BB rats. Diabetes 38:1623-1629,1989
176. Bottazzo GF, Pujol-Borrell R, Hanafusa T, Feldman M. Role of aberrant HLA-DR expression and antigen presentation in induction of endocrine autoimmunity. Lancet 2:1115-1119,1983
177. Wright JR, Hauptfeld V, Lacy PE. Induction of Ia antigen expression on murine islet parenchymal cells does not diminish islet allograft survival. Am J Pathol 134:237-242, 1989
178. Clare-Salzler M, Mullen Y. Marked dendritic cell-T cell cluster formation in the pancreatic lymph node of the non-obese diabetic mouse. Immunology 76:478-484,1992
179. Knight SC, Farrant J, Chan J, Bryant H, Bedford PH, Bateman C. Induction of autoimmunity with dendritic cells: studies on thyroiditis in mice. Clin Immunol Immunopathol 48:277-289,1988
180. Oschilewski U, Kiesel U, Kolb H. Administration of silica prevents diabetes in BB-rats. Diabetes 34:197-199,1985
181. Lee KU, Pak CY, Amano K, Yoon JW. Prevention of lymphocytic thyroiditis and insulitis in diabetes prone BB rats by the depletion of macrophages. Diabetologia 31:400-402,1988
182. Lee K-U, Amano FK, Yoon J-W. Evidence for initial involvement of macrophage in development of insulitis in NOD mice. Diabetes 37:989-991,1988
183. Ihm S-H, Yoon J-W. Studies on autoimmunity for initiation of β-cell destruction. VI. Macrophages essential for development of β-cell-specific cytotoxic effectors and insulitis in NOD mice. Diabetes 39:1273-1278,1990
184. Lefkowitch J, Schreiner G, Cormier J et al. Prevention of diabetes in the BB rat by essential fatty acid deficiency: relationship between physiological and biochemical changes. J Exp Med 171:729-743,1990
185. Hutchings P, Rosen H, O'Reilly L, Simpson E, Gordon S, Cooke A. Transfer of diabetes in mice prevented by blockade of adhesion-promoting receptor on macrophages. Nature 348:639-642,1990
186. Kolb H, Kolb-Bachofen V. Nitric oxide: a pathogenetic factor in autoimmunity. Immunology Today 13:157-160,1992
187. Krönke KD, Kolb-Bachofen V, Bersick B, Burkart V, Kolb H. Activated macrophages kill pancreatic syngeneic islet cells via arginine-dependent nitric oxide generation. Biochem Biophys Res Commun 175:752-758,1991
188. Yamada K, Nonaka K, Hanafusa T, Miyazaki A, Toyoshima H, Tavui S. Preventive and therapeutic aspects of large dose nicotinamide injections on diabetes associated with insulitis: an observation in non-obese diabetic (NOD) mice. Diabetes 31:749-753,1982
189. Ledoux SP, Hall CR, Forbes PM, Patton NJ, Wilson GL. Mechanisms of nicotinamide and thymidine protection from alloxan and streptozocin toxicity. Diabetes 37:1015-1019,1988
190. Nomikos IN, Prowse SJ, Carotenuto P, Lafferty KJ. Combined treatment with nicotinamide and desferrioxamine prevents islet allograft destruction in NOD mice. Diabetes 36:1302-1304,1986
191. Palmer JP, Helqvist S, Spinas GA, Mølvig J, Mandrup-Poulsen T, Andersen HU, Nerup J. Interaction of β-cell activity and IL-1 concentration and exposure time in isolated rat islets of Langerhans. Diabetes 38:1211-1216,1989

192. Mandrup-Poulsen T, Bendtzen K, Dinarello CA, Nerup J. Human tumor necrosis factor potentiates human interleukin 1-mediated rat pancreatic beta-cell cytotoxicity. J Immunol 139:4077-4082,1987
193. Pukel C, Baquerizo H, Rabinovitch A. Destruction of rat islet cell monolayer cultures by cytokines. Synergistic interactions of interferon-γ, tumor necrosis factor, lymphotoxin, and interleukin-1. Diabetes 37:133-136,1988
194. Soldevila G, Buscema M, Doshi M, James RFL, Bottazzo GF, Pujol-Borrell R. Cytotoxic effect of IFN-γ plus TNF-α on human islet cells. J Autoimmunity 4:291-306,1991
195. Serreze DV, Leiter EH. Defective activation of T suppressor cell function in nonobese diabetic mice. Potential relation to cytokine deficiencies. J Immunol 140:3801-3807,1988
196. Lety M-A, Coulaud J, Bens M, Dardenne M, Homo-Delarche F. Enhanced metabolism of arachidonic acid by macrophages from nonobese diabetic (NOD) mice. Clin Immunol Immunopathol 64:188-196,1992

Chapter 2

AN IMMUNOHISTOCHEMICAL STUDY ON ORGANIZED LYMPHOID CELL INFILTRATES IN FETAL AND NEONATAL PANCREASES. A COMPARISON WITH SIMILAR INFILTRATES FOUND IN THE PANCREAS OF A DIABETIC INFANT

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Chapter 2

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AN IMMUNOHISTOCHEMICAL STUDY ON ORGANIZED LYMPHOID CELL INFILTRATES IN FETAL AND NEONATAL PANCREASES

A comparison with similar infiltrates found in the pancreas of a diabetic infant

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Lymphoid cell infiltrates were analyzed using immunohistochemical techniques on 5 normal fetal and 6 normal neonatal pancreases. Data were compared to data obtained analyzing the lymphoid cell infiltrates in the pancreas of an 8 months old diabetic infant.

In the normal fetal and neonatal pancreases islets were intact and not infiltrated. In the diabetic infant β-cells had vanished in almost all islets, the remaining islets showed a minor infiltration with primarily T-cells, a few B-cells, and some classical marcrophages.

It appeared that a widespread infiltration of the exocrine pancreas with single dendritic-like cells, and T-cells, and little clusters of these cells were normal features of fetal and neonatal pancreases. In the diabetic case these infiltrative patterns were more pronounced.

Larger accumulations of such lymphoid cells could also be detected in the normal fetal and neonatal pancreases and these consisted mainly of T-cell zones, sometimes containing HEV's, with intermingled interdigitating dendritic cells and a few macrophages. This architecture is reminiscent of peripheral lymphoid tissue, such as bronchus-or gut-associated lymphoid tissue. The function of this fetal/neonatal intrapancreatic lymphoid tissue (which disappears in later life) is unknown. Various possibilities are suggested such as a yet unknown ubiquitous fetal/neonatal microbial infection, tolerance induction towards islet cell antigens, an endocrine regulatory function of infiltrated lymphoid cells, and a normal ontogenetic process.

KEY WORDS: Immunohistochemistry, lymphoid structures, pancreas, fetal, neonatal.

INTRODUCTION

It is now well accepted that Beta cell destruction in type I diabetes mellitus takes more than months and sometimes even years before clinically overt diabetes develops[1–5]. Mononuclear cell infiltration of the islets is the hallmark of this destruction, and florid insulitis has mainly been detected close to the time of diagnosis.

This report describes an immunohistochemical study on a pancreas of an 8 months old diabetic infant, who suddenly died after a short period of illness due to a ketoacidotic coma. Insulitis and remnants of islets lacking β-cells were found, but also—and more strikingly—widespread lymphoid cell accumulations in the exocrine pancreas. The phenotype of the composing lymphoid cells and the architecture of these lymphoid cell accumulations was reminiscent of that of peripheral lymphoid tissue.

In normal fetal and neonatal pancreases similar, but smaller lymphoid cell accumulations have been reported as early as 1962[6]. The function of these lymphoid cell accumulations is unknown and recent studies employing monoclonals to identify the various lymphoid cell subsets in these accumulations are lacking. We therefore carried out an immunohistochemical study on the presence of cells belonging to the monocyte-macrophage lineage, the T-cells and the B-cells in normal fetal and neonatal pancreas tissue. Data on the infiltrated cells found in the fetal/neonatal pancreases were compared to those found in the pancreas of the diabetic child.

MATERIALS AND METHODS

Normal fetal and neonatal pancreases

Pancreases were collected from 5 fetuses of 14–17 wk

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gestation and 6 neonates, ages ranging from 2 d to 8 months. The fetuses were from abortions performed on socio-economic grounds in a special clinic (van Bloemenhove Clinic, Heemstede, The Netherlands). Pancreas collection had been approved by the medical –ethical committee of our University. In 2/5 fetuses the pancreas was cut into 2 halves, in the other 3 the pancreas was too small to be cut, and was therefore frozen as a whole.

The neonates had died from several causes, such as prematurity and SIDS. Obduction was carried out either at the Department of Pathology, Free University, Amsterdam (dr. J.J. v.d Harten), or at the Department of Pathology, Dijkzigt Hospital, Rotterdam (dr. J.C. den Hollander). Parts of head and tail of the pancreases were removed and cut into blocks ($\approx$ 1 cm^3) within 6 h after death. In 3 neonates only a tail-part was obtained. The pancreas tissue from Amsterdam was fixed in sublimate formol and paraffin-embedded, that from Rotterdam was snap frozen in liquid nitrogen.

Pancreas of the diabetic child

A male 8 month old infant, the second child of the family had an uneventful delivery at 40 wk gestation. The child thrived well and there was no past or recent history of major viral infections. He nevertheless suddenly died within four days. During these four days the child had become ill, vomited and had diarrhoea but there were no signs of a respiratory tract infection. There was a polyuria although the child hardly drunk. Only the last day there was an elevated body temperature of 103°F (39.5°C). The child became tachypneic, lost consciousness and died after attempted resuscitation in a ketoacidotic coma.

Post-mortem blood chemistry showed hyperglycemia: 21.5 mmol/l glucose. Chemistry performed on the cerebrospinal fluid also showed a high glucose concentration: 46.5 mmol/l. There were no signs of infection in the cerebrospinal fluid.

Several parts of both head and tail of the pancreas were removed at autopsy 5 h after death, cut into blocks ($\approx$ 1 cm^3) and fixed in sublimate formol or snap-frozen in liquid nitrogen.

In routine histopathology it was established that the majority of islets lacked insulin-containing β-cells, while insulitis was evident in the remaining islets. The diagnosis diabetes mellitus type I was thus established postmortem and based on the clinical picture, the hyperglycemia, the almost complete absence of insulin containing β-cells, and the lymphoid infiltrates around the remaining islets.

Identification of the various cells infiltrated in the pancreas.

Serial 4 μm sections from both paraffin embedded material as well as from snap-frozen parts were cut and used for routine staining techniques and for indirect immunoperoxidase techniques to identify and characterize infiltrating lymphoid cells. The monoclonal and polyclonal antibodies used are listed in Table 1. The cryostat sections of the snap-frozen

Table 1 Monoclonal and polyclonal antibodies used in this study.

Antibody	*For the detection of*	*Source*
For cryostat sections		
Okla	HLA-DR	Orthodiagnostics
RFD1	Class II associated antigen present on dendritic cells	L. Poulter[7]
L25	all B cells, interdigitating cells	Y. Ishii[8]
HECA425	dendritic cells, HEV's	A. Duijvestjn[9]
Ki-M6 (αCD68)	phagocytosing macrophages	Behring
UCHT1 (αCD3)	all T-cells	Dakopatts
Leu4 (αCD3)	all T cells	Becton & Dickinson
Leu3 (αCD4)	helper/inducer T cells	Becton & Dickinson
Leu2 (αCD8)	cytotoxic T cells	Becton & Dickinson
B4 (αCD19)	all (precursor) B cells	Becton & Dickinson
B1 (αCD20)	all B cells	Becton & Dickinson
Leu14 (αCD22)	precursor and mature B cells	Becton & Dickinson
αIgM	IgM containing plasma cells	Dakopatts
αInsulin	Insulin containing cells	Pel Freez
αGlucagon	Glucagon containing cells	Dakopatts
αSomatostatin	Somatostatin containing cells	Dakopatts
For paraffin sections		
MT_1	all leucocytes, except B cells	Euro Diagnostics
LN_1	all B cells	Biotest
LN_2	all leucocytes, except T cells and plasma cells	Biotest
LN_3	HLA-DR	Biotest

material were acetone fixed prior to the application of the antibodies. Sections from the normal pancreases and the patient's pancreas were stained simultaneously with unrelated monoclonal antibodies and conjugates to check for the presence of a non specific binding of the materials.

The specific antibodies were incubated with the tissue sections for 45 min. The subsequent application of conjugates and second markers was always preceded and followed by a 10 min washing in 0.05 M phosphate buffered saline (pH 7.6). Antibodies were detected with either Horseradish Peroxidase labeled Rabbit anti Mouse serum or equivalents depending on the source of monoclonal antibodies using 3.3 diaminobenzidine tetra HCl (Sigma, USA) as substrate (brown). After rinsing in tap water, sections were briefly counterstained with hematoxylin.

Immunoperoxidase staining for monoclonal antibodies as Okla, RFD1, L25, and HECA 452 was combined in several occasions with a histochemical staining for acid phosphatase activity (red) to discriminate between the cells of the monocyte-macrophage lineage, including the dendritic cells. Antigen-presentation to T cells is most effectively carried out by dendritic cells. The family of the dendritic cells comprises the Langerhans cell from the skin, the veiled cells in the lymph, and the interdigitating cells from the spleen and lymph nodes[10]. The precursor of the dendritic cell originates from the bone-marrow, and cells showing immunohistological characteristics and functional characteristics of veiled cells can be matured from blood monocytes[11]. The immunohistological characteristics of the family of dendritic cells are[12]: a dendritic shape, an eccentric positioned reniform nucleus, absence of cytoplasmic acid phosphatase activity, and a strong membranc bound class II MHC positivity. The RFD1 antigen is regarded as a relative specific marker for activated dendritic cells[7,8], such as the interdigitating dendritic cells.

Classical macrophages specialized in phagocytosis were identified on the basis of their strong reactivity for Ki-M6 (a lysosome related antigen) and acid phosphatase (a lysosomal enzyme), in combination with their morphology (large, ruffled cells). The above mentioned criteria were used by us to make a distinction between antigen presenting cells of the dendritic cell family and macrophages suitable for phagocytosis.

To establish the spatial relationship between the various infiltrated lymphoid cells and the pancreatic islet cells double stainings were performed in the fetal and neonatal pancreases using B1, UCHT-1, RFD-1, and Okla in combination with the anti-insulin polyclonal antibody. The monoclonals were developed in an immunoperoxidase staining as described above, the anti-insulin antibody was developed with an APAAP-technique, as described earlier by Mason and Sammons[13].

Using a MOP-videoplan (Leitz) the following parameters were quantified: a) numbers of isolated cells with a dendritic morphology, negative acid phosphatase staining, a reniform nucleus, and strong class II positivity in the exocrine part of the pancreas, b) the percentage of surface area occupied by lymphoid tissue relative to pancreatic tissue, and c) the mean islet surface area in μm^2 in the plane of the section. For the enumeration of dendritic-like cells in the exocrine pancreas 5 randomly chosen microscopic fields (magnification ×400) per section in at least 4 different sections, cut at an interval of at least 100 μm were studied for each individual (5 fetuses/6 neonates/diabetic). For the quantification of the percentage surface area occupied by lymphoid tissue relative to pancreatic tissue at least 4 clearly different (i.e. different in architecture at low magnification) sections were studied in the 5 fetuses (in two fetuses sections from two distinct blocks) and the one diabetic child. In the neonates only 2 clearly different sections could be studied in each individual. For the mean islet surface area in μm^2 in the plane of the section 100 randomly chosen islets in at least 4 clearly different sections were studied for each individual.

Table 2 Infiltrative pattern of lymphoid cells in neonatal/fetal pancreases and in the pancreas of a diabetic child.

	Exocrine part			*Endocrine part*	
	Number of isolated dendritic cells per mm²	*Clustering of dendritic cells with T lymphocytes*	*% of surface area of the section taken in by dense lymphoid cell accumulations*	*Insulitis*	*Mean surface area in the plain of the section (μm^2) of endocrine islets (magn. ×1000) n=100*
Fetal	179 ± 57	+	1.46 ± 1.80	—	6240 ± 3268
Neonatal	161 ± 67	±	.55 ± .29†	—	10930 ± 8464
Diabetic	806 ± 249**	++	4.35 ± 2.35*	± (endstage)	2320 ± 1916**

* $p<0.05$.
** $p<0.005$.
†limited number of sections.

RESULTS

Pancreases of the 5 fetuses and 6 non-diabetic neonates.

Considerable numbers of MHC-class II positive dendritic-shaped cells were seen evenly distributed throughout the stroma of the exocrine pancreas (see Figure 1a and Table 2). These cells were weakly positive for the macrophage markers acid phosphatase and CD68 (Ki-M6). Occasionally there were large ruffled cells in the stroma clearly positive for Ki-M6 (Figure 2a) and/or acid phosphatase (Figure 2b) and negative or weakly positive for MHC-class II. We considered these latter cells as classical macrophages.

The dendritic-shaped cells, positive for MHC-class II, were sometimes present at the border of the islets (Figure 1b). In a few of these cases the cells clustered together and formed a small rim-like structure. The dendritic-like cells were never seen infiltrating the fetal or neonatal islets.

Some T-lymphocytes ($CD3^+$, round cells with a high nucleus/cytoplasmic ratio) were also present in the stromal compartment, however, very few. These T-cells were practically always seen in small cellular clusters of about 3–4 cells. T-lymphocytes or B-lymphocytes ($CD19^+$, round cells) were never seen infiltrating the islets. Hence, insulitis was completely lacking.

Besides the above described isolated dendritic-like cells evenly distributed throughout the stroma of the

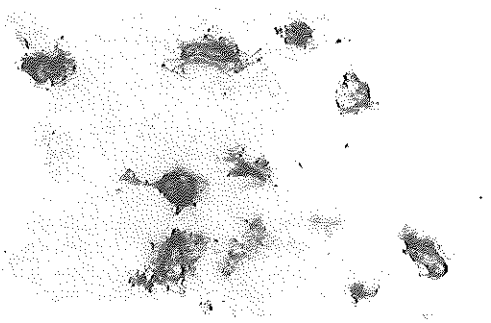


Figure 1(a) Single dendritic cells in the exocrine part of a fetal pancreas, sometimes forming small clusters. Immunoperoxidase staining for class II MHC (brown, Okla). Magnification, ×630.

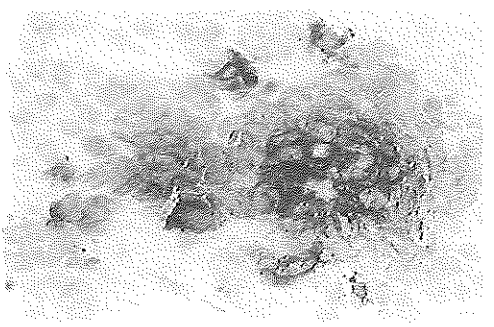
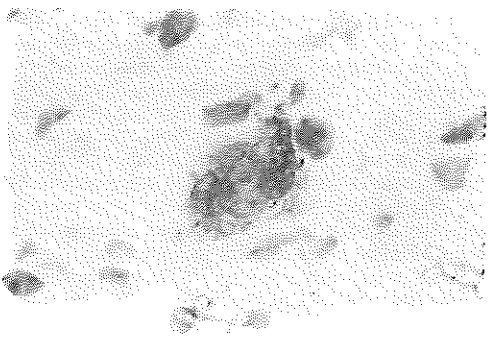
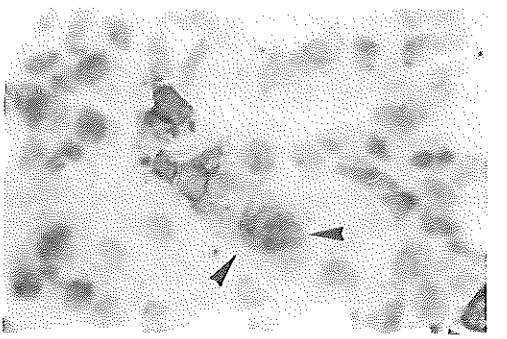


Figure 1(b) Dendritic cells (brown) in a fetal pancreas, positioned in the connective tissue in between in exocrine acini, and around a fetal islet (red = insulin). Immunoperoxidase staining with anti-class II MHC antibody (brown, Okla), and APAAP staining with anti-insulin (red). Magnification, ×630.



(a)



(b)

Figure 2 Single classical macrophages, staining strongly positive for lysosomal enzymes, present in the connective tissue in between the exocrine acini of a fetal pancreas. (a) Immunoperoxidase staining with Ki-M6 (αCD68, a lysosome related antigen, brown). Magnification, ×630. (b) Histochemical staining for acid phosphatase activity (red). Magnification, ×630.

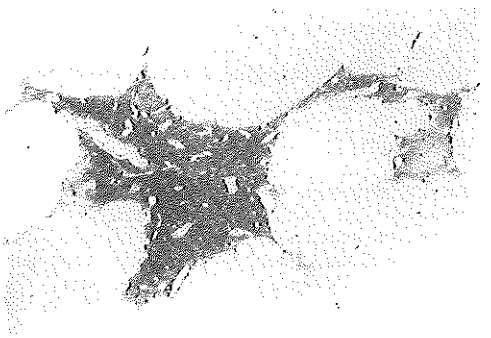


Figure 3(a) Organized lymphoid tissue in a neonatal pancreas present in the interlobular connective tissue. Immunoperoxidase staining with MT1 (brown, all leucocytes, except B-cells). In the lymphoid tissue HEVs can easily be detected (arrow). Magnification, ×63.

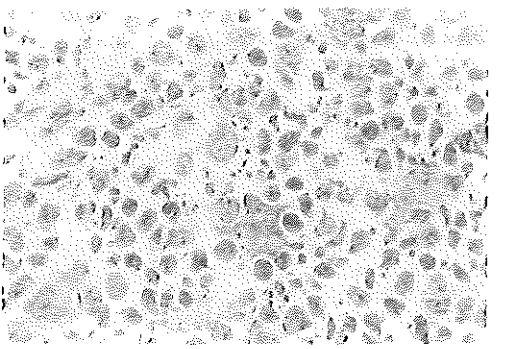


Figure 3(b) Dendritic cells present in an organized lymphoid structure found in the capsular connective tissue of a fetal pancreas. The cells show the immunohistochemical characteristics of dendritic interdigitating cells of the T-cell areas in lymph nodes and spleen (dendritic morphology, reniform nucleus, positive for RFD1). Immunoperoxidase staining with RFD1 (brown, activation marker of dendritic cells). Magnification, ×630.

exocrine pancreas and the small clusters of T cells, there were in all fetal and in 4 out of the 6 neonatal pancreases larger accumulations of lymphoid cells, showing a certain grade of organization (Figure 3a). These lymphoid structures took 1,46% (range 0,1–5,4%) in fetal pancreases and were predominantly found in the capsular and (sometimes) interlobular connective tissue compartment. In the neonatal pancreases the surface area of these lymphoid structures was 0,55% (range 0,00–0,99%) and found at the same localizations, however the number of sections studied

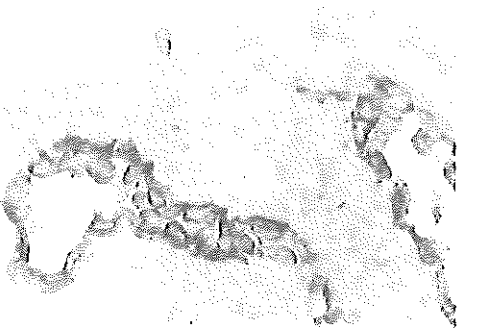


Figure 3(c) High endothelial venule (HEV) present in the T-cell zone of a lymphoid cell accumulation in the interlobular connective tissue of a neonatal pancreas. Immunoperoxidase staining with HECA 452 (brown, adhesion molecule on HEVs and some dendritic cells). Magnification, ×630.

in these pancreases is limited (see Materials and Methods). The lymphoid structures were mainly composed of T-cells intermingled with cells showing the immunohistomorphological characteristics of dendritic interdigitating cells of T cell areas in lymph nodes and spleen (namely RFD1 positive and weakly positive spots for CD68, Figure 3b). High endothelial venules (HECA 452 positive cuboid endothelial cells) were also present in the T-cell zones of the larger of these lymphoid cells accumulations (Figure 3c). HEV's are special venules characteristic of the highly organized T-cell areas of peripheral lymphoid tissues. In the larger lymphoid cell accumulations B-cells ($CD19^+$ and $LN1^+$, round cells) could be detected as well, grouped in a follicle-like structure, adjacent to the T-cell area. Moreover, in all lymphoid cell aggregates there were a few classical macrophages, viz ruffled cells that were strongly positive for acid phosphatase activity, without MHC-class II expression.

Pancreas of the diabetic child

The pancreas of the diabetic child was of normal texture and development and weighed 6.5 grams (the average weight for the gland of that age is 13±7 grams).

In immune histomorphology the diagnosis of diabetes mellitus was reconfirmed and the majority (96%) of the islets was found devoid of insulin containing cells. Only the two other hormone producing cells (viz. the somatostatin and glucagon positive cells) were found in all islets. The islet-surface in the plain of the section was significantly ($p<0.005$) smaller (mean±SD: 2320±1916 μm^2) when compared to the islet surface in the non-diabetic neonatal

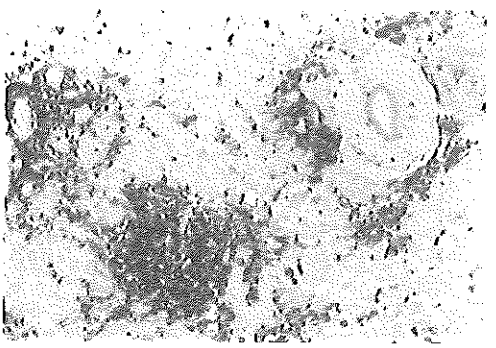


Figure 4 Organized accumulations of lymphoid cells in the diabetic pancreas situated in between the exocrine acini. Immunoperoxidase staining with MT1 (brown, all leucocytes except B-cells). Magnification, ×160.

(mean±SD: 10930±8464 μm^2) and fetal pancreases (mean±SD: 6240±3268 μm^2).

Islets still containing some beta cells showed a minor lymphoid cell infiltration. The lymphocytes infiltrating these islets were mainly of the T-helper/inducer ($CD4^+$, round cells) as well as of the T-cytotoxic ($CD8^+$, round cells) phenotype. Acid phosphatase positive macrophages, which were also positive for MHC-class II, could also be detected in the insulitis process. In the islet-infiltrations some B-lymphocytes were present as well.

In the stroma of the exocrine pancreas isolated cells with a dendritic morphology, as described in the fetal/neonatal pancreases were again present and some of them formed small cellular clusters with T-lymphocytes like in the normal fetal and neonatal pancreases. The number of these isolated dendritic cells, T-cells, and clusters of these cells in the exocrine part of the diabetic pancreas was however clearly higher than those of the neonatal and fetal pancreases (Table 2).

As in normal neonatal and fetal pancreases there were also organized accumulations of lymphoid cells in the exocrine parts of the diabetic pancreas. Though these accumulations were in principle of similar architecture as those found in the non-diabetic fetal and neonatal pancreases (see before), they were more numerous and occupied more surface area of the pancreas sections as compared to the surface area occupied in the normal neonatal and fetal tissues (Table 2). Moreover, the lymphoid tissue structures were not only present in the capsular and interlobular connective tissue compartment, they also extended to and were situated in between the exocrine acini (Figure 4). The lymphoid cell accumulations were again mainly composed of T-cells. In these T cell zones HEV's and interdigitating cells were clearly present. In some of these lymphoid cell accumulations clusters of few B-lymphocytes and a few scattered macrophages, which stained strongly for acid phosphatase but were negative for MHC class II could clearly be identified. However, fully developed B-cell follicles were absent.

DISCUSSION

Our histopathologic study shows, that a widespread infiltration with immune cells and the presence of organized accumulations of such cells are normal features of fetal and neonatal pancreases. In the diabetic infant these infiltrative patterns were more pronounced. The clinical diagnosis of diabetes in this child was proven by the absenceof insulin-producing β-cells in the vast majority of the islets of the patient. The islets with a few remaining β-cells showed insulitis, characterized by minor lymphoid cell infiltration. The islets lacking β-cells were relatively small; the mean surface area in the plane of the section was lower as compared to the mean surface area in the plane of the section of the non-diabetic neonatal and fetal pancreases. We assume that this form of insulitis represents the endstage of the disease since we have found similar patterns of minor cell infiltration in the islets of the BB-rat at the endstage of the disease[14]. Moreover, in these diabetic rats the surface area of the islets lacking β-cells was also smaller in comparison to normal healthy islets in Wistar rats.

The organized lymphoid cell accumulations present in the diabetic infant and in the normal fetal and neonatal pancreases were mainly composed of T-cell areas with HEV's, RFD1-positive interdigitating dendritic cells, as well as a few macrophages, positive for lysosomal activity. In a minority a B-cell-follicle-like structure could be detected. The architecture of these accumulations is reminiscent of that of gutmucosa-associated-lymphoid-tissue (MALT), of bronchus-associated-lymphoid-tissue (BALT) and of similar structures found in the thyroid in autoimmune thyroid disease[15,16]. However, in contrast to the intrapancreatically developed lymphoid tissue B cell follicles with fully developed germinal centers are often present in MALT, BALT, and thyroiditis[17,18].

One might wonder, what the immunological function of the intrapancreatic lymphoid tissue in fetuses and neonates is. It must be noted that these structures disappear later in life.

Firstly, the lymphoid tissue could reflect an immune reactivity directed towards an exogenous viral or bacterial antigen. This would mean, that all our normal fetuses were infected with a pancreatotropic microbe before birth; up till now such pancreatic infections have not been described.

Secondly, the presence of the lymphoid tissue might be taken as representing a regulatory immune

reaction towards self-antigens; or in other words to serve the induction of tolerance or immune suppression towards islet cell antigens. However, the function of structures with a similar architecture, e.g. bronchus-associated-lymphoid-tissue and gut-associated-lymphoid-tissue is generally regarded to serve immune stimulation[19]. On the other hand evidence has recently been put forward, that peripheral extrathymic induction of tolerance can be achieved as a consequence of an excessive immune stimulation by superantigens. The superantigen MIs[4] was capable after i.v. injection to induce tolerance by clonal deletion of $V\beta6^+$-CD4$^+$-T-cells after an initial expansion of these $V\beta6^+$ cells[20]. It is not known if some islet cell antigens are capable to cause tolerance in a similar way, and whether our lymphoid tissue represents the initial expansion of islet-reactive T-cells to become deleted later.

Thirdly, it can be hypothesized, that the infiltrated lymphoid cells serve an endocrine regulatory function. Isolated dendritic cells and macrophages can be detected in other endocrine tissues, such as the ovary and the thyroid[21-23]. These monocyte-derived cells are thought to act in the endocrine regulation of these organs via cytokine production; TNFα and Il-1 are described to exert effects on the endocrine function of pancreatic β-cells and thyrocytes[24]. Cytokines produced by T-cells also have an effect on endocrine cells[25].

Finally, the transient presence of intrapancreatic lymphoid tissue might simply reflect a process of lymphoid tissue development during the ontogeny of the pancreas. It is not known which stimuli induce the formation of lymphoid tissue in general during fetal development, and how such stimuli are programmed. In the case of the intrapancreatic lymphoid tissue this stimulus could be the presence of pancreatic ductuli, analogous to the formation of gut- or bronchus-associated-lymphoid-tissue close to the mucosa of gut or bronchus respectively. Also a specific homing of lymphoid cells to the pancreas via programmed vessels (HEV's) might play a role.

In conclusion, intrapancreatic lymphoid tissue is a normal feature during the fetal/neonatal development of the pancreas. In a diabetic child the presence of this lymphoid tissue was more pronounced at the time the majority of β-cells had vanished. The function of intrapancreatic lymphoid tissue during fetal and neonatal life is an intriguing problem, which needs further investigations.

Acknowledgements

We wish to thank Dr J.J. v.d. Harten and Dr J.C. Den Hollander for the help in obtaining the pancreases, Tar van Os for the excellent photographic work, and Rieke Elsenbroek and Annelies de Vries for secretarial assistance.

References

1. Gepts W. Pathologic anatomy of the pancreas in juvenile diabetes mellitus. *Diabetes* 1965; **14**: 619–633
2. Gepts W, De Mey J. Islet cell survival determined by morphology – an immunocytochemical study of the islets of Langerhans in juvenile diabetes mellitus. *Diabetes* 1987; **27** (suppl 1): 251–261
3. Gorsuch AN, Spencer KM, Lister J, McNally JM, Dean BM, Bottazzo GF, Cudworth AG. Evidence for a long prediabetic period in Type I (insulin-dependent) diabetes mellitus. *Lancet* 1981; **2**: 1363–1365
4. Srikanta S, Ricker AT, McCulloch DK, Soeldner JS, Eisenbarth GS, Palmer JP. Autoimmunity to insulin, beta cell dysfunction, and development of insulin-dependent diabetes mellitus. *Diabetes* 1986; **35**: 139–142
5. Powers AC, Eisenbarth GS. Autoimmunity to islet cells in diabetes mellitus. *Ann Rev Med* 1985; **36**: 533–44
6. Liu HM, Potter EL. Development of the human pancreas. *Arch Path* 1962; **74**: 439–452
7. Poulter LW, Campbell DA, Munro C, Janossy G. Discrimination of human macrophages and dendritic cells by means of monoclonal antibodies. *Scan J Immunol* 1986; **24**: 351–357
8. Ishii Y, Takami T, Kokai Y, Yuasa H, Fujimoto J, Takei T, Kikuch K. A Novel Human B-lymphocyte antigen shared with lymphoid dendritic cells: characterization by monoclonal antibody. *Clin Exp Immunol* 1985; **61**: 624–632
9. Duijvestijn AM, Horst E, Pals ST, Rouse BT, Steere A, Picker L, Meijer CJLM, Butcher EC. High endothelial differentiation in human lymphoid and inflammatory tissues defined by monoclonal antibody HECA 452. *Am J Pathol* 1988; **130**: 147–155
10. Steinman RM. The dendritic cell system and its role in immunogenicity. *Ann Rev Immunol* 1991; **9**: 271–296
11. Kabel PJ, de Haan-Meulman M, Voorbij HAM, Kleingeld M, Knol EF, Drexhage HA. Accessory cells with a morphology and marker pattern of dendritic cells can be obtained from elutriator purified blood monocyte fractions. An enhancing effect of metrizamide in this differentiation. *Immunobiology* 1989; **179**: 395–411
12. Austyn JM. Lymphoid dendritic cells. *Immunology* 1987; **62**: 161–170
13. Mason DY, Sammons R. Alkaline phosphatase and peroxidase for double immunoenzymatic labelling of cellular constituents. *J Clin Pathol* 1978; **31**: 452–460
14. Voorbij HAM, Jeucken PHM, Kabel PJ, de Haan M, Drexhage HA. Dendritic cells and scavenger macrophages in the diabetic islets of prediabetic BB rats. *Diabetes* 1989; **38**: 1623–1629
15. Plesch BEC. Histology and immunohistochemistry of bronchus associated lymphoid tissue (BALT) in the rat. *Adv Exp Med Biol* 1982; **149**: 491–497
16. Kabel PJ, Voorbij HAM, de Haan-Meulman M, Pals ST, Drexhage HA. High endothelial venules present in lymphoid cell accumulations in thyroids affected by autoimmune disease: a study in men and BB rats of functional activity and development. *J Clin Endocrinol Metab* 1989; **68**: 744–51
17. Wilkin TJ, Casey C. The distribution of immunoglobulin containing cells in human thyroiditis. *Acta Endocrinol* 1984; **106**: 490–498
18. Voorbij HAM, Kabel PJ, de Haan-Meulman M, Jeucken PHM, van der Gaag RD, de Baets MH, Drexhage HA. Dendritic cells and class II MHC expression on thyrocytes during the autoimmune thyroid disease of the BB-rat. *Clin Immunol Immunopathol* 1990; **55**: 9–22
19. Bienenstock J, Befus D. Gut- and Bronchus-associated lymphoid tissue. *Am J Anat* 1984; **170**: 437–445

20. Webb S, Morris C, Sprent J. Extrathymic tolerance of mature T cells: Clonal elimination as a consequence of immunity. *Cell* 1990; **63**: 1249–1259
21. Kabel PJ, Voorbij HAM, de Haan M, van der Gaag RD, Drexhage HA. Intrathyrodial dendritic cells. *J Clin Endocrin & Metab* 1988; **66**: 199–207
22. Adashi EY. The potential role of cytokines to ovarian physiology: The emerging role of resident ovarian cells of the white blood cell series. *Endocrine Rev* 1990; **11**: 454–464
23. Wilders-Truschnig MM, Kabel PJ, Drexhage HA, Beham A, Leb G, Eber O, Heberstreit J, Loidolt D, Dohr G, Lanzer G, Kreys GJ. Intrathyroidal dendritic cells, epitheloid cells, and giant cells in iodine deficient goiter. *Am J Pathol* 1989; **135**: 219–225
24. BendtzenK, Buschard K, Diamant M, Horn T, Svenson M. Possible role of Il-1, TNFα, and Il-6 in insulin dependent diabetes mellitus and autoimmune thyroid disease. Thyroid cell group. *Lymphokine-Res* 1989; **8**: 335–340
25. Gorospe WC, Tuchel T, Kasson BG. γ-interferon inhibits rat granulosa cell differentiation in culture. *Biochem Biophys Res Commun* 1988; **157**: 891–897

Chapter 3

IMMUNOHISTOCHEMICAL STUDIES ON MURINE PANCREASES DURING SPONTANEOUS DEVELOPMENT OF IDDM, AND AFTER VARIOUS TREATMENTS ACCELERATING OR POSTPONING THE DISEASE

Immunohistochemical Characterization of Monocytes-Macrophages and Dendritic Cells Involved in the Initiation of the Insulitis and β-Cell Destruction in NOD Mice

Annemarie Jansen, Françoise Homo-Delarche, Herbert Hooijkaas, Pieter J. Leenen, Mireille Dardenne, and Hemmo A. Drexhage

This immunohistochemical study describes the infiltration pattern of monocytes-macrophages and dendritic cells during the development of insulitis and diabetes in the NOD mouse. A panel of monoclonal antibodies (MoAbs) was used to analyze pancreases of nondiabetic (glucosuria negative) male and female NOD mice at 3, 7, 10, and 17 weeks of age. BALB/c female mice 17-weeks-old, diabetic NOD female mice 20- to 30-weeks-old, and nondiabetic NOD male mice 22-weeks-old were used as controls. Three MoAbs (viz., ER-MP23, MOMA1, and BM8) were special and appeared to identify macrophage/dendritic cell subsets that either had a characteristic infiltration pattern in the initial phases of the autoimmune reaction before T-cell infiltration or were typical for the later β-cell destructive insulitis process. *1*) Raised numbers of ER-MP23$^+$ and MOMA-1$^+$ dendritic cells/macrophages were characteristic for the initial phases of the NOD insulitis in 3-week-old mice. The cells were found in and near swollen para-insular vessels. In 7-week-old mice, these ER-MP23$^+$ and MOMA-1$^+$ cells had accumulated around the islets and were the first hematopoietic cells detectable at these spots. *2*) From 7 weeks of age onward, BM8$^+$ macrophages could be found in the para- and peri-insulitis processes. However, only in females were these BM8$^+$ macrophages found to infiltrate into the islets. In lymphoid tissues, ER-MP23 predominantly reacts with macrophages/dendritic cells present in the subcapsular and interfollicular sinuses of lymph nodes and the T-cell zones of these lymph nodes. ER-MP23 also reacts with tissue macrophages/dendritic cells. MOMA-1 reacts with the marginal metallophilic macrophages of the spleen and with sinus macrophages of the lymph node. Both populations of cells are likely to be involved in antigen presentation in lymphoid tissues as well as in the NOD peri-insulitis. BM8 in lymphoid tissues predominantly reacts with the phagocytosing macrophages present in the red pulp of the spleen. Because β-cell destruction and glucosuria almost exclusively take place in NOD females, our findings suggest that BM8$^+$ macrophage infiltration into the female islets is linked to a β-cell destructive process, either as a destructive type of infiltration or as an infiltration meant to remove the β-cell debris caused by another immune assault. *Diabetes* 43:667–675, 1994

Insulin-dependent diabetes mellitus (IDDM) results from progressive destruction of the insulin-producing cells of the islets of Langerhans (β-cells) as a consequence of a β-cell–directed autoimmune process (1). Lymphoid cell infiltration in and around the islets of Langerhans was described in various case reports concerning patients who died shortly after disease onset (2–4). Because longitudinal analysis of these events cannot be performed in humans, animal models of IDDM may be useful for studying the sequence of the autoimmune reaction.

In a previous immunohistochemical study of the BB-insulitis (5), we described that major histocompatibility complex (MHC) class II positive cells with a phenotype and morphology of antigen-presenting dendritic cells were the first lymphoid cells to accumulate around the BB islets, followed by a local cluster formation of these cells with subsequently infiltrating lymphocytes. Infiltration by acid-phosphatase positive macrophages completed the disease process.

The monoclonal antibodies (MoAbs) presently available to identify rat lymphoid cells, and in particular cells belonging to the monocyte-macrophage and dendritic cell series, are scarce and not well defined. In contrast, a larger panel of such MoAbs is available in mice (6). We decided to use these MoAbs in the mouse model for IDDM, the NOD mouse, to obtain more information regarding the infiltration of monocytes-macrophages and dendritic cells during the development of insulitis (7,8). Although marked differences are found between the diabetes of the NOD mouse and the BB rat with regard to sex distribution and histological pattern of infiltration, a number of in vivo studies underline the role of monocytes-macrophages and of dendritic cells in both models (9–14).

Immunohistological studies have been performed on the NOD mouse insulitis process but predominantly have described the presence and involvement of the various subpopulations of T-cells and B-cells (7,15); studies on macrophage infiltration are scarce (12,16). We therefore performed a sequential study on the presence of monocytes-

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IDDM, insulin-dependent diabetes mellitus; MHC, major histocompatibility complex; MoAb, monoclonal antibody; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DAB, diamino-benzidine; IDC, interdigitating dendritic cell; SER, sheep erythrocyte receptor.

TABLE 1
Antibodies used for immunohistochemical study

	Specificity	Source	Fixation technique	Blocking step	Second antibodies
MoAb					
MT4	CD4	ATCC	Acetone	Normal rabbit serum	Rabbit α rat PO
Lyt2	CD8	ATCC	Acetone	Normal rabbit serum	Rabbit α rat PO
KT3	CD3	ATCC	Acetone	Normal rabbit serum	Rabbit α rat PO
RA3.6B2	B-220 antigen	ATCC	Acetone	Normal rabbit serum	Rabbit α rat PO
F4/80	Mature macrophages except in T-cell areas absent from dendritic cells (1)	ATCC	Acetone	Normal rabbit serum	Rabbit α rat PO
ER-HR3	Subpopulation of red spleen macrophages (2)	P. Leenen	Formaldehyde	Normal rabbit serum	Rabbit α rat PO
M1/70	Mac-1, CR3	ATCC	Pararosanilin	Normal rabbit serum	Rabbit α rat PO
M3/38	Mac-2	ATCC	Acetone	Normal rabbit serum	Rabbit α rat PO
NLDC-145	Dendritic cells	ATCC	Pararosanilin	Normal rabbit serum	Rabbit α rat PO
M5/114	H-2 Ia	ATCC	Pararosanilin	Normal rabbit serum	Rabbit α rat PO
10.3.6.P	Ia^{NOD} (3)	C. Boitard	Pararosanilin	See METHODS	Rabbit α mouse PO
ER-BMDM1	Mϕ and DC aminopeptidase (4)	P. Leenen	Pararosanilin	Normal rabbit serum	Rabbit α rat PO
30G12	T200 antigen	ATCC	Acetone	Normal rabbit serum	Rabbit α rat PO
MOMA-1	Marginal metallophilic macrophages (5)	G. Kraal	Acetone	Normal rabbit serum	Rabbit α rat PO
MOMA-2	Red pulp macrophages (6)	G. Kraal	Pararosanilin	Normal rabbit serum	Rabbit α rat PO
ER-MP23	38 kD polypeptide of unknown function (7)	P. Leenen	Pararosanilin	Normal rabbit serum	Rabbit α rat PO
BM8	Macrophages with phagocytosing capacity (8)	BMA	Acetone	Normal rabbit serum	Rabitb α rat PO
M1/42	Class I MHC	ATCC	Acetone	Normal rabbit serum	Rabbit α rat PO
Polyclonal antibody					
α-insulin		Dakopats	Acetone	Normal rabbit serum	Rabbit α Guinea Pig PO
α-glucagon		Dakopats	Acetone	Normal swine serum	Swine α Rabbit PO

PO, Peroxidase labeled

1. Austyn JM, Gordon S: F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur J Immunol* 11:805–816, 1981
2. De Jong JP, Voerman JSA, Van der Sluys-Gelling AJ, Willemsen P, Ploemacher RE: A monoclonal antibody (ER-HR3) against murine macrophages: I. Ontogeny, distribution and enzyme histochemical characterization of ER-HR3 positive cells. *Cell Tissue Res* 275:567–576, 1994
3. Oi VT, Jones PP, Goding JW, Herzenberg LA: Properties of monoclonal antibodies to mouse Ig allocytes, H-2 and Ia antigens. *Curr Top Microbiol Immunol* 81:115–125, 1978
4. Leenen PJM, Melis M, Kraal G, Hoogeveen AT, Van Ewijk W: Monoclonal antibody ER-BMDM1 recognizes a macrophage and dendritic cell differentiation antigen with aminopeptidase activity. *Eur J Immunol* 22:1567–1572, 1992
5. Kraal G, Janse M: Marginal metallophilic cells of the mouse spleen identified by a monoclonal antibody. *Immunology* 58:665–669, 1986
6. Kraal G, Rep M, Janse M: Macrophages in T- and B-cell compartments and other tissue macrophages recognized by monoclonal antibody MOMA-2. *Scand J Immunol* 26:653–661, 1987
7. Leenen PJM, Melis M, Slieker WAT, Van Ewijk W: Murine connective tissue macrophages recognized by monoclonal antibody ER-MP23. Chapter 8 of PhD Thesis, Leenen P.J.M., Erasmus University Rotterdam, the Netherlands, 1989
8. Malorny U, Michelse, Sorg C: A monoclonal antibody against an antigen present on mouse macrophages and absent from monocytes. *Cell Tissue Res* 243:421–428, 1986

macrophages and dendritic cells in the NOD pancreas during insulitis development using a panel of MoAbs characterized previously in mouse lymphoid organs and bone marrow (6).

RESEARCH DESIGN AND METHODS

Male and female mice from the NOD colony bred at the Hôpital Necker, Paris, were kept under standard conditions including a specific pathogen-free environment, given food and water ad libitum, and maintained at 22 ± 1°C on a 12:12-h light:dark cycle. In these conditions the incidence of diabetes at 30 weeks of age was 50–70% in females and ~10% in males. Male and female mice were sacrificed after rapid retro-orbital puncture by cervical dislocation at 3, 7, 10, and 17 weeks of age. Diabetic NOD female mice (age 20–30 weeks), positive for glycosuria and hyperglycemia for <14 days (Glukotest and Hemoglukotest, Boehringer-Mannheim, Germany), older nondiabetic male mice (age 22 weeks), and normal female BALB/c mice (age 17 weeks) served as disease or healthy controls. The diabetic NOD controls were not treated with insulin. Each study group consisted of 5–6 mice.

Tissue preparation. The pancreas, the spleen, the mesenterial lymph nodes, and the thymus of each mouse were removed, embedded in Tissue-tek, and snap-frozen in liquid nitrogen. Tissues were stored at −80°C.

Immunohistochemistry. Before sectioning, microscopic slides were treated by a 10 min exposure to 95% ethanol with 5% diethylether, dried in air, coated with a solution of 0.1% gelatine with 0.01% chromiumalum in distilled water, and dried in air again. Thereafter, 5-μm cryosections of the stored tissue specimens were cut, dried in air overnight, and fixed with the appropriate fixatives depending on the MoAb or polyclonal antibody used (Table 1).

Acetone fixation included an exposure to acetone (pro analyse quality) for 2 min, after which the sections were rinsed three times in phosphate-buffered saline (PBS) (pH 7.4) with 0.1% (wt/vol) bovine serum albumin (BSA) for a total of 15 min.

For the pararosanilin fixation (17), 0.4 g of pararosanilin (Sigma, St. Louis) was dissolved in 10 ml of 2 M HCl by gently heating to 37°C for 4 h; filtered, and stored at 4°C for a maximum period of 1 month. Of this stock solution, 500 μl was incubated with 500 μl of 4% $NaNO_2$ for 1 min. This mixture was dissolved in 165 ml of distilled water. Before the 2-min fixation in this solution, slides were dipped quickly in PBS. After fixation, the slides were rinsed three times in PBS with 0.1% BSA as described above.

For formaldehyde fixation, slides were exposed to 4% formaldehyde in PBS for 5 min. Thereafter, slides were rinsed in PBS with 0.1% BSA as described above.

Except for the α-insulin (DAKO, Glostrup, Denmark) and α-glucagon

(DAKO) polyclonal antibodies, the α-class II MHC MoAb, and BM8 (BMA, Biomedical AG, Augst, Swiss), all MoAbs used in this study were hybridoma culture supernatants. The α-insulin polyclonal was diluted 1:250 in PBS with 1% BSA, the α-glucagon polyclonal antibody was diluted 1:100 in PBS with 1% BSA, and BM8 was diluted 1:25 in PBS with 1% BSA.

All first-step MoAbs and polyclonal antibodies were incubated for 1 h at room temperature. After a 10 min wash in PBS with 0.1% BSA, slides were incubated with 4% normal rabbit or swine serum in PBS with 1% BSA, depending on the source of the second antibody, to diminish background staining. Without washing, the slides were then incubated for 30 min at room temperature with the appropriate second antibody (all obtained from DAKO) diluted to optimal concentration in PBS with 1% BSA to which 2% normal mouse serum was added to reduce background staining. After washing two times in PBS with 0.1% BSA, the sections were further washed in PBS without any additions. Thereafter, the peroxidase label was developed by exposure to 0.05% (wt/vol) di-amino-benzidine (DAB) in PBS, with H_2O_2 0.02% for 4 min. To remove an excess of DAB, slides were washed in streaming tap water for 5 min.

In case of an additional histochemical staining for acid-phosphatase activity, slides were then covered with a mixture containing acetate buffered AS-BI phosphate as a substrate and incubated for 20–30 min at 37°C after which staining intensity was controlled visually. Before and after counterstaining with hematoxylin for 10 s, sections were rinsed in tap water for 5 min. Sections were dehydrated by an ethanol-xylene series and embedded in Depex (BDH, Poole, England).

For the staining with 10.3.6.P, the α-class II MHC ascites MoAb reactive with I^{NOD}, a slightly different protocol was used. After pararosanilin fixation as described above, the slides were incubated with unlabeled rabbit anti-mouse polyclonal antibody diluted 1:10 in PBS with 1% BSA. Sections were rinsed three times in PBS with 0.1% BSA before and after incubation with the MoAb for 1 h at room temperature. Thereafter, slides were incubated for 30 min at room temperature with peroxidase-labeled rabbit anti-mouse antibody diluted 1:500 in PBS with 1% BSA. From this point on, the same procedure was followed as described above for the other MoAbs.

The stage and severity of infiltration varied from islet to islet in individual NOD mice, as has been described before (18). To quantify our data, we decided to stage every islet in each individual mouse separately and we identified seven stages of infiltration (based on the absence or presence and the localization of macrophages, dendritic cells, and lymphocytes. For details see RESULTS.

The following stages could be identified: *stage 0*, unaffected islets with minimal infiltration (as in control BALB/c mouse); *stage I*, stage of swollen vessel structures with no increases in the number of infiltrating cells; *stage II*, stage of early para-insular accumulation of dendritic cells and macrophages; *stage III*, similar to *stage II* but also a para-insular accumulation of lymphocytes; *stage IV*, similar to *stage III* but infiltrating cells found completely surrounding the islet (para-insulitis); *stage V*, stage of lymphocyte, macrophage, and dendritic cell infiltration into the islets; and *stage VI*, the end stage with no β-cells (insulin-positive cells) detectable.

For each age- and sex-group, the number of staged Islets was at least 100 in at least five animals. In this way, a time course of the insulitis process could be plotted.

RESULTS

Staining pattern of the panel of MoAbs. In a preliminary series of experiments (hematoxylin-eosin staining), it was established that islets in pancreases of 10-week-old NOD mice varied in infiltration grade from unaffected to heavily infiltrated with inflammatory cells. To identify the different cells involved in the insulitis process, pancreases from male and female NOD mice of this age were reacted with the panel of MoAbs, listed in Table 1, that were specific in their majority for various members of the monocyte-macrophage and dendritic cell series.

All subpopulations of monocytes-macrophages and dendritic cells could be detected in the para- or peri-insulitis process, except for NLDC-145$^+$ dendritic cells. NLDC-145 also did not react with dendritic cells in the lymph nodes, spleen, and thymus of NOD mice with the fixation-staining procedure used. The MoAb gave some reactivity in the control BALB/c lymphoid tissues, particularly in the thymus. By using acetone fixation, NLDC-145$^+$ cells were more clearly detectable. However, in our hands, with this fixative dendritic cells in the lymph nodes, spleen, and pancreases of NOD mice were still negative, whereas NOD thymic dendritic cells were clearly positive.

Mac-2$^+$ and ER-HR3$^+$ cells were present, but very scarce, in the NOD para- and peri-insulitis process. The Mac-2 and ER-HR3 epitopes are unknown; Mac-2 epitopes are preferentially expressed by macrophages after stimulation with specific differentiation signals such as thioglycollate (6). ER-HR3 particularly identifies macrophages at hemopoietic sites in the bone marrow and the splenic red pulp.

F4/80, MOMA-2, Mac-1, M1/42 (MHC class I), and $αI^{NOD}$ (MHC class II) positive cells were clearly present in the para- and peri-insulitis process. However, positive cells were found in such high numbers in the insulitis process (particularly later) that patterns of infiltration with these cells were neither characteristic nor discriminative. Particularly, F4/80 stained all subclasses of macrophages/dendritic cells, and we could therefore not use this antibody for further discrimination.

ER-BMDM1 stained a small population (2–4 per islet section) of irregularly shaped cells present in the islets of normal BALB/c mice and NOD mice of every age-group studied. These cells were acid-phosphatase negative. In large islets, numbers were raised to a maximum of 5–7 per islet section during the insulitis process.

Special patterns of infiltration were, however, observed with the MoAbs ER-MP23, MOMA-1, and BM8. Using these MoAbs, it appeared possible to describe the infiltrative process over time in more detail and to detect the very early stages of the insulitis process. We therefore also decided to re-establish the staining pattern of these antibodies in lymphoid tissues from BALB/c and NOD mice.

The MoAb ER-MP23 stained a population of cells with a dendritic morphology present in low numbers around normal islets and in the normal vessel wall of both NOD mice and of control BALB/c mice. In NOD mice, numbers of ER-MP23$^+$ cells started to rise in the vessel wall in the very early stages of the insulitis process to later accumulate around the islets before any lymphocytic infiltration (Fig. 1*C*). The ER-MP23$^+$ cells were acid-phosphatase negative. Using serial sections, it appeared that the ER-MP23$^+$ cells in the vessel walls and around the islets corresponded to cells positive for MHC class II markers and for F4/80. Later in the disease process, the ER-MP23$^+$ cells predominantly stayed around the islets (Fig. 1*D* and *E*) and virtually did not infiltrate into the islet itself except in very late stages.

In lymphoid tissues, ER-MP23 clearly and strongly stained a population of acid-phosphatase negative cells in the marginal and interfollicular sinuses and the paracortical T-cell zones of peripheral lymph nodes (Fig. 3*A*). Many of these cells had long cytoplasmic veils or protrusions interdigitating between the T-cells when in the paracortex. Hence, the ER-MP23$^+$ cells very much resembled the veiled cells and the interdigitating dendritic cells (IDCs) of the paracortical T-cell areas of the peripheral lymph nodes in location and morphology. However, not all paracortical IDCs were positive for ER-MP23. Mainly, those IDCs close to the B-cell follicles were positive. Besides this intriguing pattern in the lymph node, ER-MP23 was also positive, but less strongly, on large cells with the morphology of interdigitating cells in the

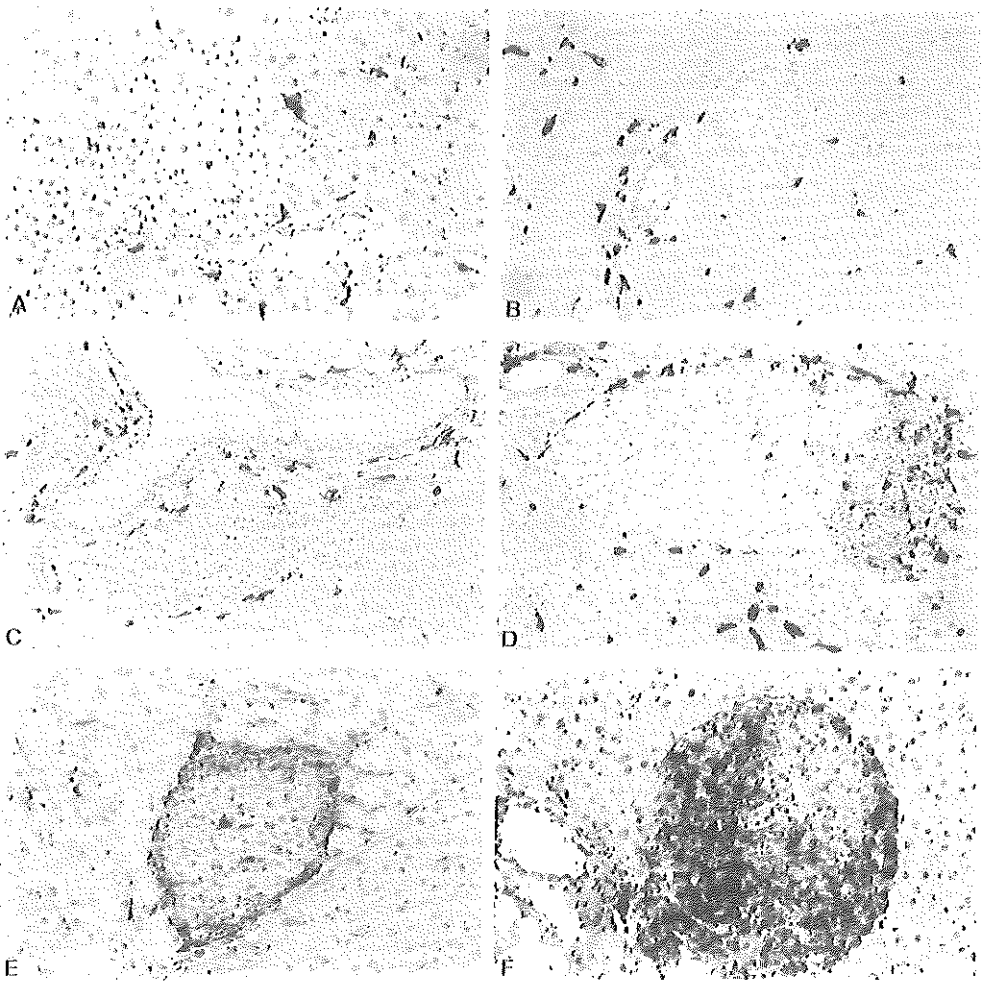

FIG. 1. Stages of infiltration, 0-V (see RESULTS for description). *A*: Stage 0, normal BALB/c islet with ER-MP23 staining, magnification ×160. Note the vessel pole with the endothelium and the small perivascular space. *B*: Stage I, islet in 3-week-old NOD female mouse with ER-MP23 staining, magnification ×160. Note the enlarged perivascular space, the higher number of ER-MP23$^+$ cells in the vessel pole, and the higher endothelium. *C*: Stage II, islet in a 7-week-old NOD female mouse with ER-MP23 staining, magnification ×160. Several ER-MP23$^+$ dendritic shaped cells can be observed in the vascular connective tissue structures and around the islet. *D*: Stage III, islet in a 10-week-old NOD female mouse with ER-MP23 staining, magnification ×250. Note the ER-MP23$^+$ cells around the islet and present in the para-insular lymphocyte accumulation. *E*: Stage IV, islet in a 17-week-old NOD female mouse with BM8 staining, magnification ×250. BM8$^+$ macrophages are in between the peri-insular lymphocyte accumulation and in the islet. *F*: Stage V, islet in a 17-week-old NOD female mouse with B220 staining, magnification ×250. Large numbers of B-cells are present in and around the islet.

peri-arteriolar lymphocyte sheets of the spleen and strongly positive on a small population of large cells in the red pulp of the spleen. These latter cells were more rounded and had short or no cytoplasmic protrusions. In the thymus, a few cells with a dendritic morphology clearly stained positive. These cells were located mainly in the outer zones of the medulla of the thymus (results not shown). ER-MP23 also stained many irregularly shaped cells present in the connective tissue capsules of the organs studied.

MOMA-1 stained an acid-phosphatase negative population of cells that were also present in the vessel walls and around normal islets of NOD mice and control BALB/c mice. Like the ER-MP23$^+$ cells, the MOMA-1$^+$ cells accumulated very early in the autoimmune process around the islets. However, the

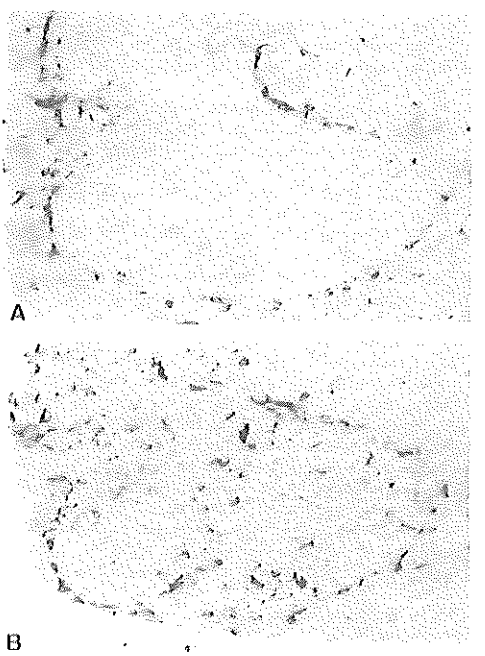

FIG. 2. Islet of Fig. 1*D*, stage IIIB in semiserial sections. *A*: MOMA-1 staining, magnification ×250. MOMA-1 positive macrophages accumulating around the islet and at the border of the para-insular lymphocytic accumulation. Note that MOMA1$^+$ macrophages do not mix with lymphocytes in the accumulation in contrast to ER-MP23$^+$ cells. *B*: BM8 staining, magnification ×250. Note the BM$^+$ macrophages infiltrating into the islet mass.

MOMA-1$^+$ cells were somewhat more rounded than the ER-MP23$^+$ dendritic-like cells. The MOMA-1$^+$ cells stayed strictly in the peri-insular compartment (Fig. 2*A*) even during the later and very late phases of the autoimmune process. In lymphoid tissue, MOMA-1 clearly and strongly stained a population of large acid-phosphatase negative cells with a ruffled membrane and long cytoplasmic protrusions in the marginal zone of the white pulp of the spleen (Fig. 3*B*). Besides this characteristic pattern, MOMA-1 also clearly stained macrophages present in the marginal sinuses of the lymph nodes and a few cells in the connective tissues. Finally, a very small population of weakly staining cells in the medulla of the thymus could be distinguished. This pattern is largely comparable with the pattern described by Kraal et al. (19).

BM8$^+$ macrophages were the first cells infiltrating into the islets during the autoimmune process (Fig. 2*B*). Of the BM8$^+$ macrophages, ~60% were strongly acid-phosphatase positive. BM8$^+$ cells infiltrating the islets were almost only seen in NOD female mice. Note that BM8$^+$ macrophages were also present in the para- and peri-insular lymphoid cell infiltrates. With regard to lymphoid tissue, BM8 strongly stained a large number of macrophages in the red pulp of the spleen (Fig. 3*C*). Of these cells, ~60% were strongly positive for acid phosphatase.

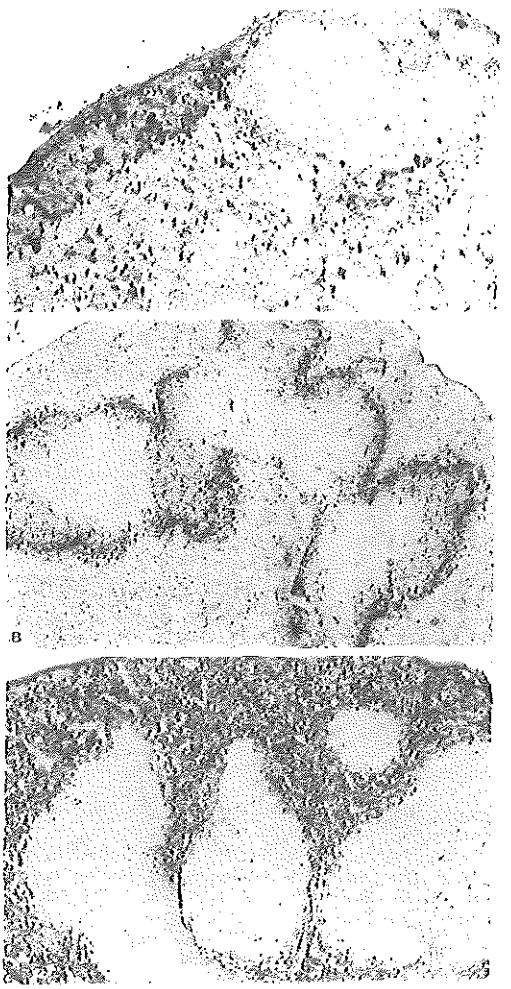

FIG. 3. *A*: ER-MP23 staining of BALB/c lymph node, magnification ×160. Note the ER-MP23 positive cells in the marginal sinuses and in the paracortical T-cell areas often close to the B-cell follicles. The B-cell follicle area is devoid of ER-MP23 cells. *B*: MOMA-1 staining of BALB/c spleen, magnification ×63. Note the characteristic pattern of the outer zonal area of the white pulp (marginal metallophilic cells). *C*: BM8 staining of BALB/c spleen, magnification ×63. Note the BM8 positivity of the macrophages of the red pulp.

Time course of the various islet infiltration patterns and stages during the NOD insulitis. Based on the pattern of MoAb positivity, seven stages of infiltration could be identified. Figure 4 represents the distribution pattern of these seven infiltration stages in 3-, 7-, 10-, and 17-week-old NOD male and female mice and their respective controls, viz, 17-week-old BALB/c mice (healthy controls) and diabetic NOD mice (disease controls).

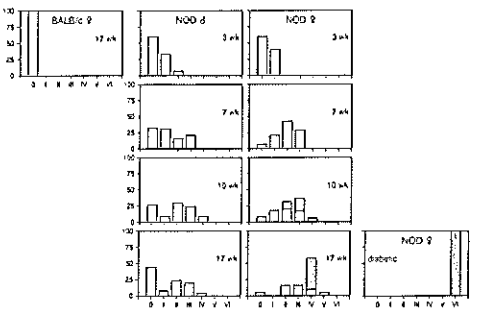

FIG. 4. Distribution (%) of types and severity of islet infiltration in the NOD mouse according to sex and age. Each sex- and age-group consisted of 5–6 mice. In each mouse, at least 20 islets were scored. Vertical hatching indicates the percentage of islets with infiltration by $BM8^+$ macrophages into the islets. See RESULTS for stages.

Stage 0 (unaffected islet). In the control BALB/c mice, no lymphocytic infiltration was observed. However, all islets contained a few ER- $BMDM1^+$ macrophages with long cytoplasmic protrusions, which lacked acid-phosphatase activity. The endothelium of the vessels adjacent to the islets was flat and the peri-vascular connective tissue compartment was small. A few ER-$MP23^+$ dendritic-like cells and MOMA-1^+ macrophages were present in this compartment, but numbers did not exceed 3–4 per islet section (Fig. 1*A*). Three-week-old NOD female and male mice (our earliest time point of study) had 60% of islets with this normal appearance. At 17 weeks of age, only 5% of the NOD islets had kept their normal appearance in nondiabetic NOD female mice. In 17-week-old NOD male mice, 44% of the islets could be considered normal, according to the characteristics described above. In the recently diabetic NOD female mice, none of the islets had a normal appearance (Fig. 4).

Stage I (swollen vessel structure). The first islet abnormality observed in NOD mice was a swelling of the vessels adjacent to islets. The endothelium became swollen and had a cubic appearance. The perivascular connective tissue compartment also became more prominent. In the wall of these swollen vessels and in the perivascular connective tissue, the number of ER-$MP23^+$ dendritic-like cells and MOMA-1^+ macrophages had increased to about twice the number of the stage 0 (unaffected) situation. No signs were found of lymphocytic infiltration. The islet itself was not affected and histologically not different from the normal BALB/c islets (Fig. 1*B*). This type of infiltration was seen predominantly in 3-week-old NOD female and male mice (40 and 33%, respectively; Fig. 4).

Stage II (stage of early para-insular accumulation of dendritic cells and macrophages). This stage in the development of NOD insulitis was characterized by an increase in the number of ER-$MP23^+$ dendritic-like cells and MOMA-1^+ macrophages mostly at the side of the islet close to the vessels (Fig. 1*C*). The cells were, however, never found to infiltrate into the islets. Besides the ER-$MP23^+$ cells and the MOMA-1^+ cells, $BM8^+$ macrophages were also present in the para-insular lymphoid cell accumulation. Lymphocytes were not present. Close to the islets with this type of dendritic cell/macrophage accumulation, a swollen vessel with raised numbers of ER-$MP23^+$ and MOMA-1^+ cells inside the vessel wall was always detected. Islets with this type of infiltration were seen predominantly in 7-week-old NOD female and 10-week-old NOD male mice (43 and 30%, respectively; Fig. 4).

Some of the stage II islets did show an infiltration into the islet by some $BM8^+$ macrophages. This infiltration into the islet mass was exclusively seen in NOD female mice. The intra-islet infiltration of $BM8^+$ macrophages was scored as a separate entity, stage IIB, and is indicated as the vertically hatched part of the bars in Fig. 4.

Stage III (para-insular accumulation of dendritic cells, macrophages, and lymphocytes). At this stage of NOD-insulitis, lymphocytes became detectable in the para-insular lymphoid cell accumulation close to the vessels. The CD4:CD8 ratio was ~1:1 in these structures. Of these para-insular accumulations of lymphoid cells, 83% also contained B-cells, which formed small B-cell clusters in 35% of the lymphocytic accumulations. MOMA-1^+ cells were now seen predominantly at the outer border of the para-insular cell accumulations (Fig. 2*A*). ER-MP23 and $BM8^+$ macrophages were present between the lymphocytes (Figs. 1*D* and 2*B*). At this stage, various other types of cells also from the monocyte-macrophage and dendritic cell family positive for the Moabs, listed in Table 1 (except NLDC145, see above), could be detected between the accumulated lymphocytes. Lymphoid cell accumulations of this type were seen primarily in 10-week-old NOD female and 22-week-old NOD male mice.

As was described for stage II islets, some of the stage III islets also showed an infiltration of $BM8^+$ macrophages into the islet mass (stage IIIB, Fig. 2*B*). Again the intra-islet infiltration of $BM8^+$ macrophages was seen almost exclusively in females (Fig. 4), apart from one older NOD male mouse (6% at 22 weeks of age, data not shown).

Stage IV (peri-insular accumulation of dendritic cells, macrophages, and lymphocytes.) The peri-insular accumulations of lymphoid cells showed the same marker pattern and architecture as was described for the para-insular (stage III) accumulations, although the islets were now surrounded completely by the dendritic cells, macrophages, and lymphocytes (Fig. 1*E*). Peri-insulitis was found predominantly in 17-week-old NOD female mice (58% of islets) and in 35% of 22-week-old NOD male mice. Also in this stage, islets could be detected showing an infiltration by $BM8^+$ macrophages into the islets. Again, this was seen predominantly in females.

Stage V (lymphocytic insulitis). At this stage, the peri-insular accumulation of lymphoid cells was still clearly present. However, this type of infiltration was characterized by massive infiltration into the islets, not only by $BM8^+$ macrophages as described above, but also accumulations of T-cells and B-cells protruded into the islet mass in high numbers (Fig. 1*F*). A few insulin-producing β-cells were still detectable. Compared with the earlier stages, the $BM8^+$ macrophages between these β-cells were found in higher numbers. ER-$MP23^+$ cells also were detected infiltrating into the islet mass. This type of infiltration was seen only in 17-week-old NOD female mice (5% of islets) (Fig. 4).

Stage VI (end stage). At this stage, insulin-producing cells were no longer detectable. However, a few glucagon-positive cells were present. The numbers of the various types of lymphocytes and macrophages infiltrating in between and surrounding the islet remnants varied from high to undetect-

able. Out of the five pancreases of diabetic females studied, one pancreas showed a few large islets composed of glucagon-positive cells without insulin-producing cells. These islets lacked any accumulation and infiltration by cells of the immune system. In hematoxylin and eosin staining, these islets could not be distinguished from normal islets. End-stage insulitis was found only in our study group of overtly diabetic NOD female mice (Fig. 4).

DISCUSSION

The staging of the NOD insulitis process used in this study and based on patterns of infiltration of various subsets of macrophages, dendritic cells, and lymphocytes differs from staging schemes used by others before. Miyazaki et al. (15) and Signore et al. (18) used conventional hematoxylin and eosin staining, and these investigators were thus able to identify only the stages involving lymphocytic infiltration, i.e., the later phases of para-insulitis, peri-insulitis, and insulitis (stages III, IV, and V in this study). Using the macrophage/dendritic cell-specific antibodies, we were able to identify very early stages of macrophage and dendritic cell infiltration without any lymphocytic infiltration (stages I and II). We were able to show that the NOD insulitis process in mice of both sexes starts with a swelling of the vessels adjacent to the islets followed by an accumulation of dendritic cells and macrophages at one side of the islet. Two subsets showed a characteristic pattern of early infiltration, i.e., ER-MP23$^+$ and MOMA-1$^+$ dendritic-like cells and macrophages. The dendritic cell/macrophage accumulation was later followed by homing of lymphocytes. Our study also shows that the actual destructive process, which occurs only in NOD female mice, is characterized by an early infiltration into the islet mass of BM8$^+$ macrophages. This process was later followed by an infiltration into the islets of lymphocytes and other types of macrophages.

The function of ER-MP23$^+$ dendritic-like cells, MOMA-1$^+$ macrophages, and BM8$^+$ macrophages may be deduced from their position in lymphoid tissues as described above and from functional studies reported in the literature (6).

ER-MP23 primarily identifies macrophages located in various connective tissues. ER-MP23 also clearly identifies a population of acid-phosphatase negative cells with dendritic protrusions in the marginal and interfollicular sinuses and the paracortical T-cell zones of peripheral lymph nodes. This latter staining pattern partly overlaps the distribution of veiled cells and IDCs, which are involved in picking up antigens, antigen transport, and antigen presentation to and stimulation of naive T-cells (20,21). Note, however, that ER-MP23 only stained a subpopulation of the IDCs in the lymph nodes close to the B-cell follicles. Because the ER-MP23$^+$ dendritic-like cells accumulating around the NOD islets were also MHC class II positive, we suggest that these cells are antigen-presenting cells and involved in the presentation of insular antigens to T-cells. In our hands, the cells were not positive for NLDC-145, which was considered by others as a dendritic cell-specific marker (NLDC-145 reacted strongly with thymus IDCs). Because contacts occurred between the ER-MP23$^+$ cells and β-cells, it can be visualized that insular antigens can be picked up by the accumulated dendritic cells. Although T-cell activation takes place primarily in the pancreas-draining lymph node of the NOD mouse (22). Therefore, the early para-insular–accumulated dendritic cells likely traveled to these lymph nodes to present the autoantigens there. Later in the process they might present the antigens directly to locally accumulated lymphocytes.

MOMA-1 characteristically reacts with macrophages situated in a rim at the margin of the white pulp of the spleen (the marginal zone). MOMA-1$^+$ cells have been identified as marginal metallophilic macrophages (19), but the function of the marginal metallophilic macrophages is not yet clear. Indications suggest that these cells are involved in antigen processing and presentation of antigens to B-cells in T-independent type II responses (23). However, these indications are based on studies in neonatal mice in which the immune system is not fully developed. Studies in adult mice did not confirm these findings. With regard to MOMA-1, it also has been described that the staining of sheep erythrocyte receptor (SER)-4, an antibody raised against the mouse macrophage-restricted hemagglutinin SER, was indistinguishable from MOMA-1 staining in the spleen (24). SER-4 was found to be involved in the binding of activated T-cells and B-cells to the marginal zone (25). Moreover, it also has been described that treatment of mice with high doses of lipopolysaccharide resulted in a decrease in the number of MOMA-1$^+$ macrophages and B-cells in the marginal zone, together with an appearance of such cells in the splenic follicles (26,27). The above observations could be indicative that MOMA-1$^+$ macrophages play a role in the stimulation of B-cells and in the regulation of B-cell migration. We like to suggest that the MOMA-1$^+$ cells accumulating around the NOD islets are involved in a similar action.

Macrophages strongly positive for BM8 are found predominantly in the red pulp of the spleen. BM8 appears to be a differentiation antigen, coming to expression late in the differentiation of macrophages. BM8 is also positive on Kupffer cells in the liver and on the inflammatory macrophages of granulomata. All these cells are characterized by their strong capacity of nonspecific phagocytosis. However, although all BM8$^+$ macrophages are phagocytosing, BM8 does not stain the heavily phagocytosing cells in the marginal zone of the spleen and the subcapsular sinuses of the lymph nodes (28). Nevertheless, it is suggestive that the BM8$^+$ macrophages infiltrating early into the NOD islets are involved in phagocytosis of islet cell or β-cell components. From this study and from the literature, it is not clear whether the BM8$^+$ macrophages invade the islets after the β-cells have died from another immune-mediated event and islet remnants need to be cleared or whether the β-cells are actually destroyed by aggressive immune-stimulated BM8$^+$ macrophages entering the islets. Macrophages are capable of destroying β-cells via interleukin-1 action and NO production (29).

A few ER-BMDM1$^+$ dendritic cells were distinguished in all normal BALB/c islets and in all NOD islets. Allaerts et al. (30) described the presence of ER-BMDM1$^+$ stellate cells in another endocrine tissue, the anterior pituitary of two separate mice strains. Stellate-shaped cells in the pituitary play a role in the regulation of hormone secretion of surrounding endocrine cells by virtue of their cytokine production (interleukin-6). The ER-BMDM1$^+$ cells in the islets may play a similar role.

The process of early accumulation of ER-MP23$^+$ dendritic cells, MOMA-1$^+$ macrophages, and BM8$^+$ macrophages

around the NOD islets was preceded by swelling of the vessels. This vascular reaction thus seems to trigger the para- and peri-insular lymphoid cell accumulation. One can only speculate on the possible mechanisms of this vascular reaction in the NOD mouse.

1. The NOD islets may aberrantly release factors or produce an excess of metabolites that stimulate a vascular reaction, which in turn would trigger an enhanced adhesion of the dendritic cell and macrophage populations. The swollen vessels very much resemble in appearance the high endothelial venules, which can be detected in the T-cell areas of peripheral lymph nodes and are involved in homing of T-cells by enhanced adherence for subpopulations of these lymphocytes (31).

2. The pancreatic vessels of the NOD mouse may be intrinsically abnormal, resulting in an abnormal infiltration with dendritic cells and macrophages. Abnormal perivascular spaces have been observed in the NOD mouse thymus (32), and pancreatic venular defects have been reported in the BB-rat (33).

3. The vascular abnormalities may be caused by abnormal functioning of the few dendritic cells and macrophages normally present in the vessel walls. Female NOD peritoneal macrophages do have an enhanced arachidonic acid metabolism (34), and defects in the differentiation and function of antigen-presenting cells have been described in the NOD mouse (35,36).

The presence of para- and peri-insulitis in the NOD males, which virtually are not affected by β-cell destruction, clearly shows that this lymphoid cell accumulation around the islets is not harmful. Thus far, it has not been possible to differentiate histologically the female (para- or peri-) insulitis from the male (para- or peri-) insulitis process in NOD mice. In our study, a significant difference between female and male insulitis was the migration into the islets of the $BM8^+$ macrophages. The number of male NOD mice showing this intra-insular infiltration by $BM8^+$ macrophages remained below 10%, and we suggest that this type of infiltration in male islets represents the onset of diabetes in a few males. Note that male NOD mice do possess $BM8^+$ macrophages, but these cells do not infiltrate into the islets. Why these phagocytosing macrophages invade the female islets might thus become a key question in understanding β-cell destruction in diabetes.

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REFERENCES

1. Powers AC, Eisenbarth GS: Autoimmunity to islet cells in diabetes mellitus. *Annu Rev Med* 36:533–544, 1985
2. Botazzo GF, Dean BM, McNally JM, Mackay EH, Swift PGF, Gamble DR: In situ characterization of autoimmune phenomena and expression of HLA molecules in the pancreas in diabetic insulitis. *N Engl J Med* 313:353–360, 1985
3. Foulis AK, Liddle CN, Farquharson MA, Richmond JA, Weir RS: The histopathology of the pancreas in type I (insulin-dependent) diabetes mellitus: a 25-year review of deaths in patients under 20 years of age in the United Kingdom. *Diabetologia* 29:267–274, 1986
4. Jansen A, Voorbij HAM, Jeucken PHM, Bruining GJ, Hootjkaas H, Drexhage HA: An immunohistochemical study on organized lymphoid cell infiltrates in fetal and neonatal pancreases: a comparison with similar infiltrates found in the pancreas of a diabetic infant. *Autoimmunity* 15:31–38, 1993
5. Voorbij HAM, Jeucken PHM, Kabel PJ, De Haan M, Drexhage HA: Dendritic cells and scavenger macrophages in pancreatic islets of prediabetic BB rats. *Diabetes* 38:1623–1629, 1989
6. Leenen PJM: *Phenotypical Analysis of Murine Macrophage Differentiation*. PhD thesis, Erasmus University Rotterdam, the Netherlands, 1989
7. Makino S, Kunimoto K, Muraoka Y, Mizushima Y, Katagiri K, Tochino Y: Breeding of a nonobese diabetic strain of mice. *Exp Anim* 29:1–13, 1980
8. Kikutani H, Makino S: The murine autoimmune diabetes model: NOD and related strains. *Adv Immunol* 51:285–322, 1992
9. Lee KU, Amano K, Yoon JW: Evidence for initial involvement of macrophage in development of insulitis in NOD mice. *Diabetes* 37:989–991, 1988
10. Charlton B, Bacelj JA, Mandel TE: Administration of silica particles or anti-Lyt 2 antibody prevents β-cell destruction in NOD mice given cyclophosphamide. *Diabetes* 37:930–935, 1988
11. Ihm SH, Yoon JW: Studies on autoimmunity for initiation of β-cell destruction. VI. Macrophages essential for development of β-cell specific cytotoxic effects and insulitis in NOD mice. *Diabetes* 39:1273–1278, 1990
12. Hutchings P, Rosen H, O'Reilly L, Simpson E, Gordon S, Cooke AV: Transfer of diabetes in mice prevented by blockade of adhesion-promoting receptor on macrophages. *Nature* 348:639–642, 1990
13. In't Veld PA, Pipeleers DG: In situ analysis of pancreatic islets in rats developing diabetes: appearance of nonendocrine cells with surface MHC class II antigens and cytoplasmic insulin immunoreactivity. *J Clin Invest* 82:1123–1128, 1988
14. Appels B, Burkatt V, Kantwerk-Funke G, Funda J, Kolb-Bachofen V, Kolb H: Spontaneous cytotoxicity of macrophages against pancreatic islet cells. *J Immunol* 142:3803–3808, 1989
15. Miyazaki A, Hanafusa T, Yamada K, Miyagawa J, Fujino-Kurihara H, Nakajima H, Nonaka K, Tarui S: Predominance of T lymphocytes in pancreatic islets and spleen of prediabetic nonobese diabetic (NOD) mice: a longitudinal study. *Clin Exp Immunol* 60:622–630, 1985
16. Reddy S, Liu W, Elliott RB: Distribution of pancreatic macrophages preceding and during early insulitis in young NOD mice. *Pancreas* 8:602–608, 1993
17. De Jong JP, Voerman JS, Leenen PJ, Van der Sluijs-Gelling AJ, Ploemacher RE: Improved fixation of frozen lympho-haemopoietic tissue sections with hexazotized pararosaniline. *Histochem J* 23:392–401, 1991
18. Signore A, Pozzilli P, Gale EAM, Andreani D, Beverley PCL: The natural history of lymphocyte subsets infiltrating the pancreas of NOD mice. *Diabetologia* 32:282–289, 1989
19. Kraal G, Janse E: Marginal metallophilic macrophages of the mouse spleen identified by a monoclonal antibody. *Immunology* 58:665–669, 1986
20. Austyn JM: Lymphoid dendritic cells. *Immunology* 62:161–70, 1987
21. Steinman RM: The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* 2:71–96, 1991
22. Clare-Salzer M, Mullen Y: Marked dendritic cell T-cell cluster formation in the pancreatic lymph node of the nonobese diabetic mouse. *Immunology* 76:478–484, 1992
23. Kraal G, Janse M, Claassen E: Marginal metallophilic macrophages in the mouse spleen: effects of neonatal injections of MOMA1 autoantibody on the humoral immune response. *Immunol Lett* 17:139–144, 1988
24. Crocker PR, Gordon S: Mouse macrophage hemagglutinin (sheep erythrocyte) receptor with specificity for sialated glycoconfugates characterized by a monoclonal antibody. *J Exp Med* 169:1333–1346, 1989
25. Kraal G: Cells in the marginal zone of the spleen. *Int Rev Cytol* 132:31–74, 1992
26. Groeneveld PHP, Erich T, Kraal G: The differential effects of bacterial lipopolysaccharide (LPS) on splenic non-lymphoid cells demonstrated by monoclonal antibodies. *Immunology* 58:285–290, 1986
27. Groeneveld PHP, Erich T, Kraal G: In vivo effect of LPS on B lymphocyte subpopulations: migration of marginal zone–lymphocytes and IgD-blast formation in the mouse spleen. *Immunobiology* 170:402–411, 1985
28. Maloerny U, Michels E, Sorg C: A monoclonal antibody against an antigen present on mouse macrophages and absent from monocytes. *Cell Tissue Res* 243:421–428, 1986
29. Mandrup-Poulsen T, Helqvist S, Wogensen LD, Molvig J, Pociot F, Johannsen J, Nerup J: Cytokines and free radicals as effector molecules in the desctruction of pancreatic beta cells. *Curr Top Microbiol Immunol* 164:169–193, 1990
30. Allaerts W, Jeucken PHM, Hofland LJ, Drexhage HA: Morphological, immunohistochemical, and functional homologies between pituitary folliculo-stellate cells and lymphoid dendritic cells. *Acta Endocrinol* 125 (Suppl. 1):92–97, 1991
31. Salmi M, Granfors K, Leirisalo-Repo M, MacDermott R, Leino R, Havia T, Jalkanen S: Selective endothelial binding of interleukin-2–dependent human T-cell lines derived from different tissues. *Proc Natl Acad Sci USA* 89:11436–11440, 1992
32. Savino W, Boitard C, Bach JF, Dardenne M: Studies on the thymus in

nonobese diabetic mouse. I. Changes in the microenvironmental compartments. *Lab Invest* 64:405–417, 1991
33. Majno G, Joris I, Handler ES, Desemone J, Mordes JP, Rossini AA: A pancreatic vernular defect in the BB/Wor rat. *Am J Pathol* 123:210–215, 1987
34. Lety M-A, Coulaud J, Bens M, Dardenne M, Homo-Delarche F: Enhanced metabolism of arachidonic acid by macrophages from nonobese diabetic (NOD) mice. *Clin Immunol Immunopathol* 64:188–196, 1992
35. Serreze DV, Gaskins HR, Leiter EH: Defects in the differentiation and function of antigen-presenting cells in NOD/Lt mice. *J Immunol* 150:2534–2543, 1993
36. Langmuir PB, Bridgett MM, Bothwell ALM, Crispe IN: Bone marrow abnormalities in the nonobese diabetic mouse. *Int Immunol* 5:169–177, 1993

Chapter 3.2

EFFECT OF PROPHYLACTIC INSULIN TREATMENT ON THE NUMBER OF ANTIGEN PRESENTING CELLS AND MACROPHAGES IN THE PANCREAS OF NOD MICE IS THE PREVENTION OF DIABETES BASED ON ß-CELL REST?

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SUMMARY

Prophylactic insulin treatment has been shown to have beneficial effects in type 1 diabetes, both in humans and in various animal models of the disease. In experimental models, the protective effect of prophylactic insulin treatment was observed in two parameters: 1) progression of the insulitis and 2) diabetes incidence. However, the mechanism of the protection still remained to be investigated. We therefore analyzed by immunohistochemistry the effect of prophylactic insulin treatment vs placebo treatment (vehicle) from 4 to 13 weeks of age on the dendritic cell/macrophage infiltration in and around pancreatic islets in the non-obese diabetic (NOD) mouse, a spontaneous model for type 1 diabetes. NOD-*scid/scid* and BALB/c mice were used as diabetes-free controls. Using conventional hematoxylin-eosin staining, we detected a protective effect of prophylactic insulin treatment in NOD females on the lymphocytic insulitis, significant at 13 weeks, but not at 9 weeks of age. However, when assessed by immunostaining for early islet infiltration of $BM8^+$ cytotoxic/phagocytosing macrophages, the reduction in severity of insulitis could already be detected as early as 9 weeks of age. With regard to the early accumulating ER-$MP23^+$, dendritic, antigen-presenting cells, we observed that their numbers per mm^2 surface area of the exocrine pancreas and per µm at the circumference of the islet were higher in placebo-treated NODs (197 ± 13.8 and 14 ± 0.9, respectively) and in NOD-*scid/scids* (227.3 ± 14.4 and 8.7 ± 0.7, resp.) as compared to age-matched BALB/c mice (123.1 ± 7.1 and 3.5 ± 0.9, resp.). Prophylactic insulin treatment of NODs lowered the attraction of ER-$MP23^+$ dendritic cells to the exocrine pancreas and to the circumference of the islets (156.3±8.5 and 7.9±1, resp.). Interestingly also, the islet size was found to be larger in placebo-treated NODs and NOD-*scid/scids* (51% and 38 were larger than 10 μm^2 in each group, respectively), than in age-matched BALB/c mice (9% larger than 10 μm^2). Prophylactic insulin treatment of NODs reduced their islet size to sizes found in the control BALB/c strain.
In conclusion, the decrease in islet size by early insulin administration, and the lower attraction of ER-$MP23^+$ dendritic-shaped cells to the islets are strong morphological indications that prevention of diabetes development by prophylactic insulin treatment results from a downregulation of islet metabolism, with a concomitant decline in the release of islet factors attracting APCs

INTRODUCTION

Insulin-dependent diabetes mellitus (IDDM) is currently believed to be an autoimmune disorder, which causes complete destruction of the insulin-producing cells, the β-cells of the islets of Langerhans[1,2,3]. The natural history of IDDM or type 1 diabetes indicates a prolonged period, termed as "pre-diabetes" with no evidence of glucose intolerance, while an active process, slowly destroying β-cells, is underway. The clinical symptoms of hyperglycemia are recognized when the β-cell mass has been reduced below 80 of the original number[2,3]. Insulin administration at this time point not only corrects the hyperglycemia, but is also associated with the recovery of endogenous insulin production, which is called the "honeymoon phase" of IDDM[4]: the pancreatic function may therefore improve when the β-cells are relieved from the hyperglycemic stress. However, such remissions are usually not permanent. More recently, the improvement of β-cell function has been confirmed in a randomized trial of intensive insulin therapy in newly diagnosed IDDM patients, using a close-loop glucose instrument, the Biostator, during the induction phase of the treatment[5]. Moreover, recurrent exposure to the Biostator was shown to decrease ICA and IAA levels[6], suggesting that the autoimmune process may cease before total β-cell destruction has occurred.

Long-term goals of type 1 diabetes research have been to develop strategies for early identification and prevention of IDDM. In this line it has been shown that prophylactic insulin treatment of subjects at high risk of developing IDDM significantly delayed the onset of diabetes[7,8]. The same improvement was observed in different animal models of type 1 diabetes, either induced by neonatal streptozocin[9], or the spontaneous models, the BB rat[10,11,12] and the NOD mouse[13]. In both the BB rat and the NOD mouse prophylactic insulin treatment not only prevented spontaneous disease, but also transfer of diabetes to young pre-diabetic animals was inhibited[1415].

In each case a slowing down of the insulitis process was observed, the mechanism of which still remains to be elucidated. Several possible mechanisms have been proposed in literature, involving actions on β-cell metabolism and/or the immune system: Firstly, slowing down of the insulitis process could be the result of a specific tolerance induction towards insulin by the subcutaneous administration of exogenous insulin, thus acting as a tolerogen. A second possible mechanism is the suggested immunosuppressive effect of insulin on the immune system[16]. Furthermore, it could also be that exogenous insulin administration downregulates β-cell metabolism[17], and quiescent β-cells express fewer antigens[18].

There is presently increasing evidence for a role of dendritic cells and macrophages at early points in the disease process, as demonstrated by several approaches in the various models[19,20,21]. In the NOD mouse we recently reported that the insulitis is preceded by enlargement of the peri-vascular area and endothelial swelling of the islet-vasculature, in combination with an accumulation of antigen presenting cells (APC), more specifically, ER-MP23$^+$, MHC-class II $^+$ cells with dendritic morphology, and MOMA1$^+$ macrophages[22,23,24]. Both cell types were the first to accumulate around the islets, before any lymphocyte infiltration. Moreover, high numbers of ER-MP23$^+$ dendritic shaped cells were also detected in the exocrine pancreas before and during the insulitis process in NOD mice. After initial accumulation of APCs around the islets, the classical lymphocytic para/peri-insulitis developed. During this later phase, BM8$^+$ macrophages infiltrated into the islet mass itself, particularly in NOD females, which are known to present a higher incidence of diabetes, as observed in most colonies[25,26]. In NOD males, however, intra-islet infiltration by BM8$^+$ macrophages was rarely observed, or appeared very late. On the basis of these results, we suggested that intra-islet infiltration by BM8$^+$ macrophages, with well-known cytotoxic/phagocytosing properties[27] was an early marker of more destructive stages of the insulitis process.

We thus hypothesized that if prophylactic insulin treatment induces β-cell rest, resulting in a reduction of autoantigen expression[18,28] and release of pro-inflammatory factors from the β-cells, lower numbers of APCs should be attracted around the islets at early stages of the disease, before any lymphocyte infiltration. As a result lower numbers of cytotoxic/phagocytosing macrophages should be detectable in later stages. Moreover, such treatment may also have measurable effects on β-cell mass and β-cell insulin content. We therefore analyzed the effects of prophylactic insulin treatment on the size of the islets of Langerhans, the early accumulation of ER-MP23$^+$ dendritic shaped cells around the islets and in the exocrine pancreas, the development of para/peri-insulitis and the intra-islet infiltration by BM8$^+$ phagocytosing macrophages. We also compared the early APC infiltration patterns and the islet characteristics observed in our colony of NOD mice to those found into non diabetes-prone strains, the BALB/c strain and the NOD-*scid/scid* strain. The scid mutation has been transferred from C.B.-17/Sz-*scid/scid* mice onto the NOD/Lt inbred strain background[29]. This genetic manipulation resulted in a NOD mouse without functional T and B lymphocytes and which, lacking the specific part of the immune system, does nor develop diabetes. It nevertheless has functioning APCs and macrophages and, as we presumed, islets with the metabolic features of the classical NOD mouse.

MATERIALS AND METHODS

NOD-mice, NOD-scid/scid mice and BALB/c-mice

Female NOD-mice were bred and kept at the facilities of the Hôpital Necker, Paris, France under specific pathogen free conditions,as has been desribed previously[26]. They were given free access to food and water. Under these conditions the prevalence of diabetes in females was around 80% at 30 weeks of age.

NOD-*scid/scid* mice were bred and kept at the Jackson Laboratory facilities. Housing conditions and development of the NOD-*scid/scid* stock have extensively been described previously[29]. BALB/c mice were bred and kept at the facilities of the department of Immunology, Erasmus University, the Netherlands.

Insulin treatment

Thirty-three female NOD-mice from the Paris colony were included into two experimental groups: 1) fourteen mice were treated with Protamine-Zinc-insulin (Organon, Oss, the Netherlands). From 4-6 weeks of age with 0.25 U Insulin per day, from the 7th week onwards with 0.50 U insulin per day. Another nineteen mice served as control and were treated with the same quantity of vehicle-fluid (placebo). The above described treatment schedule is almost similar to the protocol which induced significant reduction in diabetes incidence in the study described by Atkinson[13]. However, we did not increase the dosage of insulin after the 10th week of age to 0.75 U insulin/day, since some mice became severely hypoglycemic on this dosage of insulin and few of them died in a previous experiment. All mice were weighed and assessed for hypoglycemia at 3, 6, 9, and 13 weeks of age. Blood glucose levels were evaluated using Haemoglukotest test-tapes and quantitatively measured using colorimetry by Reflolux (Boehringer-Mannheim). Animals with glycemia above 11 mmol were classified as overtly diabetic. We observed that prophylactic insulin treatment until 13 weeks of age did not alter the growth of the NOD females as compared to the placebo-treated group. Mice that became diabetic in each group during the time of the study were not included in immunohistochemical analysis.

Preparation of the pancreases

At the age of 9 weeks 6 NOD-females from the two experimental groups (insulin vs placebo) were killed by cervical dislocation after rapid retro-orbital puncture. For detailed immunohistochemistry pancreases were taken out, embedded in Tissue-tek and frozen in n-hexane on dry-ice chilled alcohol. Tissues were stored at -80°C until immunohistochemistry was performed.

The remaining NOD-females from both experimental groups were killed at 13 weeks of age, for insulitis scoring only. The pancreases of these NOD female mice were prepared for scoring of lymphocytic insulitis only. They were excised and fixed in Bouin's Fluid, followed by 10% formalin, and were processed for paraffin embedding. Then, specimens were sectioned (4μm) at four non-contiguous levels (4-50 μm apart).
Four control, untreated NOD-scids were killed at the age of 7-9 weeks, four BALB/c mice were killed at 9 weeks of age. Pancreases of these animals were also prepared for immunohistochemistry.

Lymphocytic insulitis
Lymphocytic insulitis was assessed on a hematoxylin-eosin (H+E) staining by scoring every islet separately in one of the following classes: a) normal islet: lymphocytes are not visible on H+E staining at the edge of the islet, b) para/peri-insulitis: lymphocytes visible at one side or surrounding the islet, c) infiltrative insulitis: lymphocytes detectable infiltrating the islet, islet structure disturbed.

Monoclonal and polyclonal antibodies used to identify different types of macrophages and β cells
ER-MP23[22], to identify MHC-class II^+ APCs with a dendritic morphology, was a rat-anti-mouse hybridoma culture supernatant cultured at our department and used undiluted. BM8[27], to identify phagocytosing/cytotoxic macrophages) was obtained from BMA, Augst, Switzerland. This rat-anti-mouse monoclonal antibody was used diluted 1:25 in phosphate-buffered saline (PBS) with 1% bovine serum albumin (BSA). Both markers were detected with horseradish peroxidase (HRP) labeled rabbit-anti-rat immunoglobulins (DAKO).
Guinea-pig anti-insulin polyclonal antibody (DAKO) was used diluted 1:175 in PBS with 1% BSA, and detected with HRP labeled rabbit-anti-guinea pig immunoglobulins (DAKO).

Immunohistochemistry.
Immunohistochemistry was generally performed as described in detail in a previous report[22]. In short the following procedure was performed: Before sectioning, microscopic slides were coated with a solution of 0.1% gelatine/0.01% chromiumalum in distilled water. Thereafter, 5 μm cryo-sections of the stored tissue specimens were cut, dried in air overnight, and fixed by either pararosaniline (ER-MP23) or acetone (BM8 and α-insulin).
Before exposure to the first step monoclonal or polyclonal antibodies pancreases were preincubated for 10 minutes with 4% (v/v) normal rabbit serum. Without washing first

step monoclonal or polyclonal antibodies were applied and incubated for 1 hr at room temperature. Thereafter sections were washed three times in PBS with 0.1% BSA for a total of 15 minutes. To diminish background staining 1% of normal mouse serum was added to the HRP-labeled second step antibodies diluted in PBS with 1% BSA. This mixture was allowed to react for 10 minutes and was then applied to the cryosections. After 30 minutes of incubation at room temperature, sections were washed three times in PBS. Thereafter the HRP label was developed for 4 minutes by 0.05% DAB/0.02% H_2O_2 dissolved in PBS. Excess of DAB was removed by streaming tap water. After counterstaining by hematoxylin the slides were dehydrated by an ethanol/xylene series and covered with Depex.

Quantification of ER-MP23$^+$ dendritic-shaped cells relative to the circumference of the islets, the quantification of the infiltration of ER-MP23$^+$cells in the exocrine pancreas, the measurement of the surface area of the islets, and the quantification of the number of nuclei/μm^2 of islet surface area

The number of ER-MP23$^+$ dendritic-shaped cells with a visible nucleus at the circumference of all islets without lymphocytic para/peri-insulitis was counted in random sections in 16-41 islets per group (6 animals per group for the insulin- and placebo-treated NODs, 4 animals per group for BALB/c and NOD-*scid/scid* mice). In the groups with a low variation in the number of ER-MP23$^+$ dendritic-shaped cells at the circumference of the islets (BALB/c and insulin-treated NODs), a low number of islets (n = 16) sufficed for an accurate estimation; in the groups with a higher variation (placebo-treated NODs and NOD-*scid/scids*) more islets were judged (41 and 36, respectively). Since only islets without para/peri-insulitis could be assessed a second series of sections was made of the placebo-treated NODs to be able to judge enough islets. The length of the circumference of all assessed islets in the plane of the section was measured in the same sections using the Vidas-RT image-analysis system (Kontron Elektronik GmbH/Carl Zeiss, Weesp, the Netherlands). The number of ER-MP23$^+$ dendritic-shaped cells per µm islet circumference of each islet was computed. In each animal the number of ER-MP23$^+$ cells infiltrated into the exocrine pancreas was counted by the Vidas-RT system in 3-4 fields not including islets. The surface area of islets in the plane of the section was also measured by this image analysis system.

For the measurement of the surface area of islets, only islets without lymphocyte accumulation at the circumference of the islet, and islets with only a few lymphocytes present at the circumference, were measured. In BALB/c mice 32 islets were measured in 4 animals, in the other groups 47 to 68 islets were assessed in 4 (NOD-*scid/scid*) and 6 animals (insulin and placebo) per group.

For the number of nuclei per μm^2 islet surface area 22 random islets in 4 animals per group were assessed, using the Vidas-RT system for counting the nuclei, and simultaneous measurement of the assessed islet surface area.

BM8 infiltration into the islets, in relation to severity of lymphocytic para/peri-insulitis
All islets in the pancreas sections of each individual mouse from the two experimental groups (at least 35) were scored for infiltration by $BM8^+$ macrophages. BM8 infiltration was scored positive, if at least one $BM8^+$ macrophage, with a visible nucleus, was detectable in the plane of the section of the islet. Of each islet lymphocyte accumulation at the edge of the islet or infiltration into the islet was scored simultaneously.

Statistical analysis
Comparison of the difference between the various groups of mice for the number of $ER\text{-}MP23^+$ dendritic-shaped cells per µm circumference of the islet, the number of $ER\text{-}MP23^+$ cells/mm^2 exocrine pancreas, the percentage of islets in individual mice infiltrated by $BM8^+$ macrophages, and the insulitis classifications of the two experimental groups were performed with the Mann-Whitney, two sample two-tailed signed rank test. Comparison of the number of nuclei per μm^2 islet surface area in the different experimental groups was performed with the two-sample, two-tailed student's t-test.

RESULTS

Severity of insulitis in insulin and placebo treated NODs
Table 1 shows that infiltrative insulitis had hardly progressed at 9 weeks of age, affecting about 15% of the islets in both experimental groups. Though the number of intact islets was higher and that with para/peri-insulitis was lower in the insulin-treated group than in the placebo-treated group, the differences were not statistically significant at 9 weeks of age. However, prophylactic insulin treatment until 13 weeks of age protected the islets of Langerhans as reflected by the significant increase in intact islets associated with the decrease in islets with infiltrative insulitis ($p = 0.02$ and $p = 0.005$, respectively) in the insulin-treated group as compared to the placebo-treated group.

Table 1 Effect of prophylactic insulin treatment on the severity of insulitis (lymphocytic insulitis) in NOD females as assessed by H + E staining

experimental group	n	insulitis classification % of islets (m±sem)		
		intact islets	para/peri-insulitis	infiltrative insulitis
placebo, 9 wks	6	27.7 ± 3.6	56.7 ± 4.7	15.7 ± 5.6
insulin, 9 wks	6	30.8 ± 13.8	36.0 ± 12.9	13.4 ± 6.2
		p=0.18	p=0.43	p=0.93
placebo, 13 wks	13	20.3 ± 6.9	4.1 ± 1.1	75.6 ± 7.0
insulin, 13 wks	8	45.7 ± 8.5	11.5 ± 5.1	42.8 ± 5.2
		p=0.02	p=0.3	p=0.005

n = number of mice

Effect of insulin-treatment on islets in NODs. Comparison with islets of NOD-scid/scid and BALB/c mice.

There was no immunohistochemical evidence for a lower insulin-content of the β-cells (fainter staining) in either one group, being it insulin- or placebo-treated NODs, NOD-*scid/scids* or BALB/c mice. What was remarkable was, that the placebo treated NODs - the group that developed the most severe insulitis- presented larger islets at 9 weeks of age, as compared to age-matched BALB/c mice (fig 1). In placebo-treated NODs 51% of the islets was larger than 10 μm^2 in the plane of the section, and 11% was even larger than 30 μm^2; in BALB/c mice, only 9% of the islets had a surface area in the plane of the section larger than 10 μm^2, there were no islets found to be larger than 30μm^2 (difference statistically significant, $p<.0001$, using Fisher's exact test with the cut-off point for comparing large and small islets set at 10 μm^2).

Interestingly, insulin treatment of the NODs reduced the number of islets with a surface area larger than 10 μm^2 to numbers found in the BALB/c, and the statistical difference between NODs and BALB/c mice disappeared ($p=0.99$, see fig 1 and 2); only 9% of the islets in insulin-treated NODs was larger than 10 μm^2 at 9 weeks of age. It is worthy to note that in NOD-*scid/scids* the number of large and very large islets was comparable to placebo treated NODs ($p=0.186$): 38% of the islets was larger than 10 μm^2, and 9% was larger than 30 μm^2 (see fig.1).

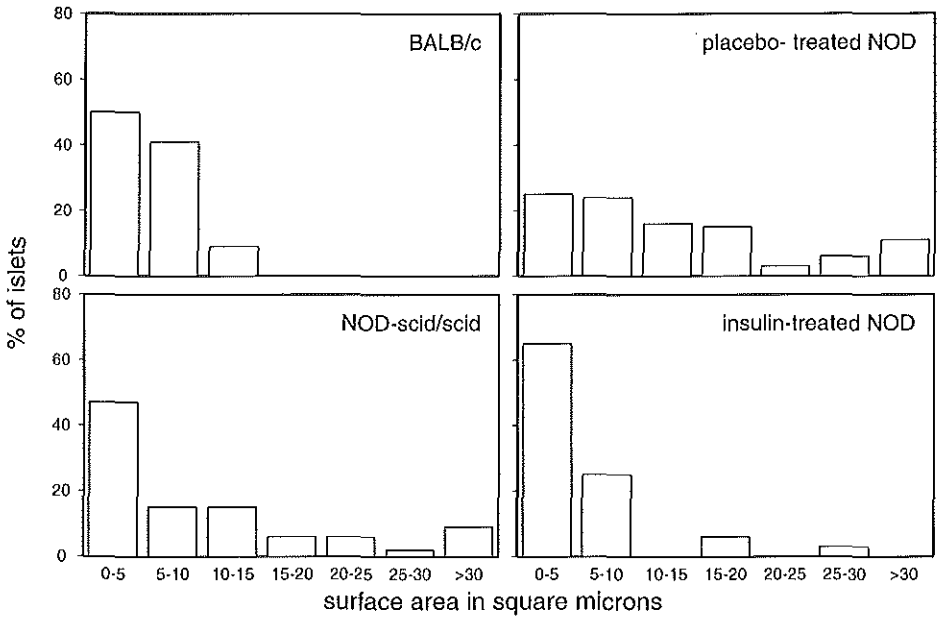

Figure 1. Distribution of islet sizes in placebo-treated NODs and insulin treated NODs, as compared to islet sizes in BALB/c and NOD-*scid/scid* mice. x-axis represents the islet sizes from small to large, y-axis the percentage of islets found with a corresponding islet size. Note that the placebo-treated NODs and NOD-*scid/scid* have large islets, as compared to BALB/c mice. Insulin treatment reduces islet sizes to sizes comparable to BALB/c islets.

Increase in islet size may be the result of β-cell hyperplasia or β-cell hypertrophia or both. On the mechanism by which insulin-treatment induces small-sized islets can only be speculated. We therefore counted the number of nuclei per μm^2 of islet surface area. It was found that BALB/c mice have a significantly lower number of nuclei per surface area, than NOD mice, being it either placebo-treated or insulin-treated NOD mice, or NOD-*scid/scids* ($p<0.05$, table 2). In spite of the reduction in islet size, insulin-treatment did not alter the number of nuclei per μm^2 in islets, as compared to placebo-treated NOD mice ($p=0.18$).

Effect of insulin treatment on the numbers of ER-MP23$^+$ dendritic-shaped cells in the exocrine pancreas and around the islets in NODs as compared to numbers in NOD-scid/scids and BALB/c mice

In control BALB/c mice 123.1±7.1 ER-MP23$^+$ cells, infiltrated per mm^2 exocrine pancreas were detectable at 9 weeks of age (table 3). At the circumference of the islets 3.5±0.9 ER-MP23$^+$ dendritic-shaped cells were found per μm circumference. The number of ER-MP23$^+$ cells in placebo-treated NODs was significantly higher than in BALB/c mice both in the exocrine pancreas ($p=0.0006$) and around islets without

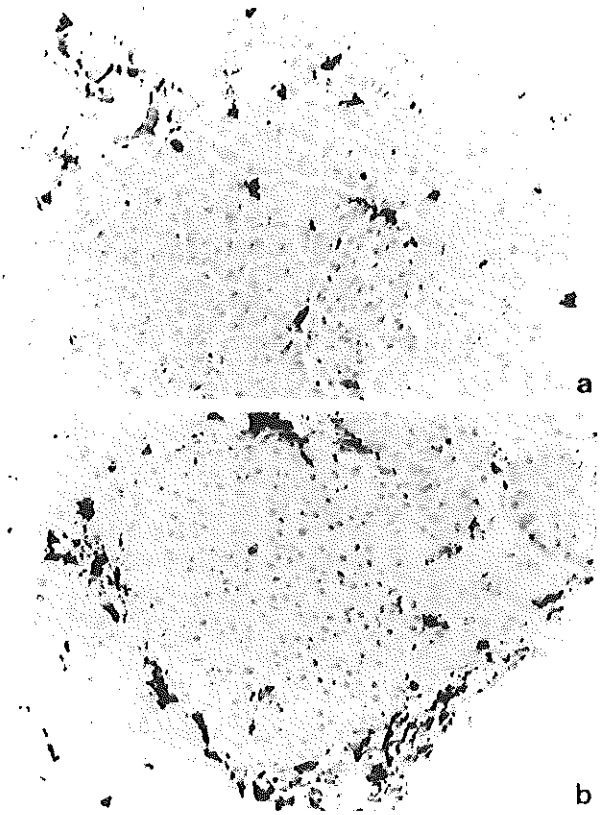

Figure 2. Islet in (a) insulin-treated female NOD of 9 weeks of age, and (b) placebo-treated female NOD of 9 weeks of age. ER-MP23 staining, magn 250x (in both photos). Note the considerable difference in size. Note also that the number of ER-MP23$^+$ cells at the edge of the islet is reduced in the insulin treated NOD.

para/peri-insulitis ($p<0.0001$) at 9 weeks of age.

Prophylactic insulin treatment significantly reduced the number of ER-MP23$^+$ cells in the exocrine pancreas ($p=0.05$) and around the islets ($p=0.0003$) as shown in table 3 and fig 2. However, insulin treatment in this study did not completely reduce the numbers of ER-MP23$^+$ cells in the exocrine pancreas ($p=0.015$) and around the islets ($p=0.0035$) to levels which were found in the control BALB/c.
It is of interest to note that 7 to 9 weeks old NOD-*scid/scids* exhibited comparable numbers of ER-MP23$^+$ in the exocrine pancreas as the placebo-treated NODs ($p=0.14$);

the number of ER-MP23$^+$ dendritic-shaped cells accumulated around the islets was also higher in the NOD-*scid/scid* as compared to BALB/c, but were not as high as in the placebo treated NODs ($p < .0001$, table 3).

Table 2 Number of β-cells per μm^2 of islet surface area in the different groups of mice

experimental groups	n	number of β-cells per μm^2 (m±std)	statistical significance as compared to BALB/c mice
BALB/c	20	4.2 ± 1.3	
NOD-*scid/scid*	22	5.8 ± 1.6	p=0.0004
placebo-treated NOD	22	5.0 ± 0.9	p=0.03
insulin-treated NOD	22	5.3 ± 0.9	p=0.002

n = number of islets analyzed
comparison of placebo-treated versus insulin-treated NOD: p= 0.2

Effect of insulin treatment on the infiltration of BM8$^+$ phagocytotic/cytotoxic macrophages into islets relative to insulitis score at 9 weeks of age.

When severity of insulitis was assessed by BM8$^+$ macrophage infiltration into the islets, a significant reduction in severity of insulitis could already be recognized at 9 weeks of age ($p=0.008$, table 4) in islets with a detectable para/peri-insulitis. Note that at 9 weeks of age, signs of infiltrative lymphocytic insulitis could hardly be recognized on H+E sections (table 1).

It is worthy to mention, that in the diabetes-free BALB/c and NOD-*scid/scid* neither infiltration of BM8$^+$ macrophages into the islets, nor an accumulation of BM8$^+$ macrophages around the islets was seen (table 4).

Table 3 ER-MP23$^+$ cell infiltration into the pancreas and around the islets in the different groups of mice

experimental group	age	n_1	n_2	number of ER-MP23$^+$ cells in the exocrine pancreas (m±sem/mm^2)	number of ER-MP23$^+$ cells at the edge of islets (m±sem/µm circumference)
BALB/c	9 weeks	4	16	123.1 ± 7.1	3.5 ± 0.9
NOD-*scid/scid*	7-9 weeks	4	36	227.3 ± 14.4	8.7 ± 0.7
placebo treated NODs	9 weeks	6	41	197.0 ± 13.8	14.0 ± 0.9
insulin treated NODs	9 weeks	6	16	156.3 ± 8.5	7.9 ± 1.0

n_1 = number of mice per group
n_2 = number of islets analyzed

main statistical significances: exocrine pancreas placebo-treated NOD versus BALB/c: $p = 0.0006$
insulin- versus placebo-treated NOD: $p = 0.05$
around the islets placebo-treated NOD versus BALB/c: $p < 0.0001$
insulin- versus placebo-treated NOD: $p = 0.0003$

Table 4 BM8$^+$ macrophage infiltration in the islets mass in the different groups of mice

experimental group	age	n	BM8 infiltration into islets respective to insulitis state % of islets (m±sem)	
			with no visible lesion	with detectable lymphocytes
BALB/c	9 wks	4	nil	nil
NOD-*scid/scid*	7-9 wks	4	nil	nil
placebo-treated NOD	9 wks	6	24.4±7.7	70.0±7.5
insulin-treated NOD	9 wks	6	6.7±4.2	46.6±3.1
			p=0.13	p=0.008

n = number of mice

DISCUSSION

This immunohistochemical study underlines the role of two different components in the pathogenesis of IDDM: first the differential involvement of two types of dendritic cells/macrophages in the progression of insulitis in the NOD mouse, and second the existence of intrinsic abnormalities of the islets of Langerhans in both the NOD and the NOD-*scid/scid* strains. In NOD mice, both components are modulated by prophylactic insulin treatment.

With regard to the progression of the insulitis in the NOD mouse, we confirmed the effect of prophylactic insulin treatment in lowering the severity of destructive insulitis[13,14]. Moreover, we showed that if the effect of the treatment on the infiltrative lymphocytic insulitis, as assessed by H+E staining was not detectable at 9 weeks of age, it became clearly visible and significant at 13 weeks of age. However, our more detailed immunohistochemical analysis detected some effects of the prophylactic insulin treatment, already visible at 9 weeks of age, consisting of a decrease in the number of accumulating antigen-presenting ER-MP23$^+$ cells around the islets and into the pancreas, before any detectable lymphocytic involvement, associated with a reduction in the number of cytotoxic/phagocytosing BM8$^+$ macrophages in islets with already detectable para/peri-insulitis. In the NOD-*scid/scid* model, ER-MP23$^+$ dendritic-shaped cells were also observed in the exocrine pancreas and in the islets of Langerhans at values significantly higher than those found in the BALB/c strain, taken as control. However, BM8$^+$ macrophages did not appear spontaneously attracted to the pancreas of NOD-*scid/scid* mice. The differential involvement of the two dendritic cell/macrophage cell types in both strains with an NOD background may be of functional significance, as discussed below, in the light of the other observation of this study, related to the size of the islets of Langerhans.

In fact, it should be underlined that, like normal NODs[30] and in contrast to BALB/c mice, NOD-*scid/scids* exhibited large-sized islets. These data suggest that intrinsic islet abnormalities are related to the NOD mouse background, and preceded the appearance of the autoimmune reaction, classically assessed by the appearance of lymphoid cells in the para/peri-insulitis and destructive insulitis. The functional significance of the differential involvement of ER-MP23$^+$ and BM8$^+$ cells may be analyzed in the context of these islet abnormalities: more ER-MP23$^+$ cells with an APC-function are attracted to the pancreas and the islets in both strains with large-sized islets, the NOD and the NOD-*scid/scid*, than in the control BALB/c strain. Since NOD-*scid/scids* lack functional T and B lymphocytes, this step is not followed neither by the classical autoimmune reaction,

the insulitis, nor by diabetes development. Moreover, the fact that NOD-*scid/scid* islets did not attract $BM8^+$ macrophages, in contrast to NOD mouse islets, suggests that attraction of $BM8^+$ cells is linked to the activation of the specific, lymphocyte-related part of the immune system. NODs and NOD-*scid/scids* show a higher number of nuclei per μm^2 islet surface area, suggesting that the large-sized islets in NODs are mainly the result of β-cell hyperplasia, in the absence of a total outgrowth of the β-cells, giving rise to large islets with small β-cells. The origin of this β-cell hyperplasia in mice with the NOD background remains to be investigated.

Finally it is also of importance to note that prophylactic insulin treatment resulted in a smaller size of the islets of Langerhans in NOD mice (comparable to the size found in BALB/c mice). The number of β-cells per μm^2 islet surface area was not altered by insulin treatment. Thus, though the size of the islets in insulin-treated NODs was comparable to BALB/c islets, the number of β-cells per islet was higher, the size of the β-cells being comparable to placebo-treated NODs. This indicates that NODs may intrinsically have smaller β-cells than BALB/c mice. Insulin treatment prevents excessive β-cell hyperplasia, but permits the production of a normal amount of β-cell mass.
The reduction in islet-size was associated with a lower number of infiltrative cells into the pancreas and around and into the islets. This results is similar to that described in BB rats[18,31], in which transplantation of the insulin-producing RINm38 insulinoma cell line resulted in smaller islets with more quiescent β-cells with a lower expression of β-cell antigens. In fact, transient hyperactivity of the β-cells has been observed in different situations leading to IDDM[32]. This observation led to the hypothesis that the functional sate of the β-cells plays an important role in the pathogenesis of type 1 diabetes. Indeed, a number of in vitro experiments has shown that active β-cells, producing high amounts of insulin are more susceptible to killing by immune products, than quiescent β-cells[32,33]. Moreover, higher β-cell activity has been associated with higher antigen expression[18,28]. In vivo, the importance of a low insulin-output by β-cells is further underlined by the fact that in BB rat diazoxide administration, which decreases insulin secretion, reduces the incidence of diabetes[34]. Since insulin administration is well known to inhibit endogenous insulin secretion[17], essentially via an indirect neural control[35,36], our data also probably result from β-cell rest.

REFERENCES

1. Powers AC, Eisenbarth GS. Autoimmunity to islet cells in diabetes mellitus. Annu Rev Med 36:533-544,1985
2. Bach JF. Insulin-dependent diabetes mellitus as an autoimmune disease. Endocrine Rev 15:516-542,1994
3. Malone JI, Shah SC. The role of exogenous insulin in the pathogenesis of autoimmune diabetes mellitus. Pediatr Adolesc Endocrinol 23:135-139,1993
4. Agner T, Damm P, Binder C. Remission in IDDM: Prospective study of basal c-peptide and insulin dose in 268 consecutive patients. Diabetes care 10:164-169,1987
5. Shah SC, Malone JI, Simpson NE. A randomized trial of intensive insulin therapy in newly diagnosed insulin-dependent diabetes mellitus. New Eng J Med 320:550-554,1989
6. Malone JI, MacLaren NK, Riley W, Spillar R. High dose insulin therapy (HDIT) resulted in long term survival of beta cells and disappearance of islet cell antibodies (ICA) in IDDM. Diabetes 40:152A,1991
7. Keller RJ, Eisenbarth GS, Jackson RA. Insulin prophylaxis in individuals at high risk of type I diabetes. Lancet 341:927-928,1993
8. Ziegler A-G, Bachmann W, Rabl W. Prophylactic insulin treatment in relatives at high risk for type 1 diabetes. Diabets Metab Rev 9:289-293,1993
9. Portha B, Picon L. Insulin treatment improves the spontaneous remission of neonatal streptozotocin diabetes in the rat. Diabetes 31:165-169,1982
10. Gotfredsen CF, Buschard K, Frandsen EK. Reduction of diabetes incidence of BB Wistar rats by early prophylactic insulin treatment of diabetes-prone animals. Diabetologia 28:933-935,1985
11. Gottlieb PA, Handler ES, Appel MC, Greiner DL, Mordes JP, Rossini AA. Insulin treatment prevents diabetes mellitus but not thyroiditis in RT6-depleted diabetes resistant BB/Wor rats. Diabetologia 34:296-300,1991
12. Vlahos WD, Seemayer TA, Yale J-F. Diabetes prevention in BB rats by inhibition of endogenous insulin secretion. Metabolism 40:825-829,1991
13. Atkinson MA, Maclaren NK, Luchetta R. Insulitis and diabetes in NOD mice reduced by prophylactic insulin therapy. Diabetes 39:933-937,1990.
14. Thivolet CH, Goillot E, Bedossa P, Durand A, Bonnard M, Orgiazzi J. Insulin prevents adoptive cell transfer of diabetes in the autoimmune non-obese diabetic mouse. Diabetologia 34:314-319,1991
15. Bertrand S, Paepe M, Vigeant C, Yale J-F. Prevention of adoptive transfer in BB rats by prophylactic insulin treatment. Diabetes 41:1273-1277,1992
16. Hunt U, Eardley DD. Suppressive effects of insulin and insulin like growth factor 1 (IGF-1) on immune responses. J Immunol 136:3994-3999,1986.
17. Argoud GM, Schade DS, Eaton RP. Insulin suppresses its own secretion in vivo. Diabetes 36:959-962,1987.
18. Appel MC, Dotta F, O'Neil J, Eisenbarth GS. Beta cell activity regulates the expression of islet antigenic determinants. Diabetologia 32:461A,1989
19. Lee KU, Amano K, Yoon JW. Evidence for initial involvement of macrophage in development of insulitis in NOD mice. Diabetes 37:989-991,1988
20. Voorbij HAM, Jeucken PHM, Kabel PJ, de Haan M, Drexhage HA. Dendritic cells and scavenger macrophages in pancreatic islets of prediabetic BB rats. Diabetes 38:1623-1629,1989
21. Ihm SH, Yoon JW. Studies on autoimmunity for initiation of ß-cell destruction. VI. Macrophages essential for development of ß-cell specific cytotoxic effects and insulitis in NOD mice. Diabetes 39:1273-1278,1990
22. Jansen A, Homo-Delarche F, Hooijkaas H, Leenen PJ, Dardenne M, Drexhage HA. Immunohistochemical characterization of monocytes-macrophages and dendritic cells involved in the initiation of the insulitis and β-cell destruction in NOD mice. Diabetes 43:667-675,1994
23. Kraal G, Janse M. Marginal metallophylic macrophages of the mouse spleen identified by a monoclonal antibody. Immunology 58:665-669,1986
24. Kraal G, Janse M, Claassen E. Marginal metallophylic macrophages in the mouse spleen: effects of neonatal injection of MOMA-1 antibody on the humoral immune response. Imm Lett 17:139-144,1988
25. Pozzilli P, Signore A, Williams AJK, Beales P. NOD mouse colonies around the world - recent facts and figures. Immunology Today 14:193-196,1993
26. Fitzpatrick F, Lepault F, Homo-Delarche F, Bach J-F, Dardenne M. Influence of castration, alone or combined with thymectomy, on the development of diabetes in the nonobese diabetic mouse. Endocrinology 129:1382-1390,1991
27. Mallorny U, Michels E, Sorg C. A monoclonal antibody against an antigen present on mouse macrophages and absent from monocytes. Cell Tissue Res 243:421-428,1986

28. Aaen K, Rygaard J, Josefsen K, Petersen H, Brogren C-H, Horn T, Buschard K. Dependence of antigen expression on functional state of ß-cells. Diabetes 39:697-701,1990
29. Prochazka M, Gaskins HR, Shultz LD, Leiter EH. The nonobese diabetic scid mouse: model for spontaneous thymomagenis associated with immunodeficiency. Proc Natl Acad Sci USA 89:3290-3294,1992
30. Serreze DV, Leiter EH. in: Insulin-dependent diabetes mellitus in NOD mice and BB rats: origins in hematopoietic stem cell defects and implications for therapy. Chap 7 from: Lessons from animal diabetes. V. Ed. E Shafrir, 1994, Smith-Gordon, GB
31. Chick WL, Warren S, Chute RN, Like AA, Lauris V, Kitchen KC. A transplantable insulinoma in the rat. Proc Natl Acad Sci USA 74:628-632,1977
32. Buschard K. The functional state of the beta cells in the pathogenesis of insulin-dependent diabetes mellitus. Autoimmunity 10:65-69,1991
33. Palmer JP, Helqvist S, Spinas GA, Mølvig J, Mandrup-Poulsen T, Andersen HU, Nerup J. Interaction of β-cell activity and Il-1 concentration and exposure time in isolated rat islets of Langerhans. Diabetes 38:1211-1216,1989
34. Vlahos WD, Yale J-F. Prevention of type I diabetes in the BB rat by diazoxide. Clin Invest Med 10:B65,1987
35. Strubbe JH, Steffens AB. Neural control of insulin secretion. Horm Metab Res 25:507-512,1993
36. Boden G, Chen X, DeSantis R, Kolaczynski J, Morris M. Evidence that suppression of insulin secretion by insulin itself is neurally mediated. Metabolism 42:786-789,1993

Chapter 3.3

THE DIABETES RETARDANT EFFECT OF INTRATHYMIC IMPLANTATION OF SYNGENEIC ISLETS IN NOD/LT MICE IS ASSOCIATED WITH REDUCED ISLET INFILTRATION BY PHAGOCYTOTIC MACROPHAGES

A preliminary report

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INTRODUCTION

Injection of syngeneic islets into the thymus has been described to prevent or retard the development of autoimmune diabetes in NOD mice and BB-rats[1,2].
Despite this prevention of glucosuria, leucocytic infiltration is still recognizable around the islets in these intrathymically (IT)-injected NODs. A variable, but mostly low insulitis score is described for 24 weeks old NOD-females IT-injected with islets[1]. The insulin content of these islets appeared to be well preserved in these IT-injected NOD-females, despite the presence of lymphocytic insulitis in these pancreases, as assessed by H+E staining. The model of intrathymic injection of syngeneic islets suggests that variants of para/peri-insulitis exist, which are not harmful to β-cells (protective para/peri-insulitis).

We recently reported the importance of phagocytotic/cytotoxic macrophages in the destructive insulitis process of NODs: well before the infiltration of the islets by lymphocytes, $BM8^+$ macrophages could be detected in the islet mass of mice at high risk of developing glucosuria (particularly in female NOD mice[3] and in NOD mice of colonies with a high incidence of diabetes[4]). Infiltration of $BM8^+$ macrophages does not occur in the diabetes-free NOD-*scid/scid*[5], which lacks functional B and T cells[6], showing the importance of the specific immune reaction for the infiltration of this subset of macrophages.

In this short communication we report an investigation showing the absence of $BM8^+$ macrophages in NOD islets affected by para/peri-insulitis after IT-injection of syngeneic islet cells.

MATERIALS AND METHODS

Intrathymic injections of islet-cells
Syngeneic islet isolation, preparation of islet cell suspensions, and thymus injections were performed as described previously[1]. At the age of 4 weeks both thymus lobes of 5 NOD/Lt females were injected with islet cells from 100 islets/lobe (=200 islets/thymus), suspended in 0.01ml of PBS. Control NOD/Lt females (n=4) were injected with 0.01ml PBS in each thymus lobe. Mice were bred and kept at The Jackson Laboratory facilities under conditions described previously[6].

Preparation of pancreases
At 8 weeks of age all mice were killed by cervical dislocation, preceded by bleeding from the retro-orbital sinus. Pancreases were removed, and snap-frozen in isopentane,

chilled by dry-ice. Tissues were stored at -80°C until sectioning.

Staining procedures

5µm cryosections were made on gelatine/chromiumalum coated slides. After overnight drying to air, sections were either stained with hematoxylin and eosin, or were fixed by 2 min of acetone exposure, after which immunohistochemical staining for the BM8 antigen was performed as described previously[3]. In short this procedure included a preincubation with 4% normal rabbit serum, followed by incubation with BM8 monoclonal antibody (BMA, Augst, Switzerland) diluted 1:25 in PBS with 1% BSA. After washing, the monoclonal was detected by horseradish-peroxidase labeled rabbit-anti-rat polyclonal antibody (DAKO). The peroxidase label was developed with DAB by routine procedures. Excess of DAB was removed by streaming tap water. A brief counterstaining with hematoxylin was followed by dehydration in an ethanol-xylene series, and a coverslip with Depex.

assessment of the presence of lymphocytes

On H+E stained sections all islets (n=41-70) sampled from three non-overlapping pancreatic levels were examined and the respective percentages for islets without lymphocytes visible at the perimeter, islets with para/peri-insulitis, and islets with detectable infiltration of leukocytes into the islets were determined, as described previously[7]. Results are expressed as mean values ± standard deviation of these percentages.

Analysis of infiltration patterns of BM8$^+$ macrophages

For the best comparison of differences in infiltration pattern of BM8$^+$ macrophages, animals from the 2 experimental groups with comparable distributions of the different patterns of insulitis were analyzed. In this way, 3 animals from the IT-islet-injected group and 2 animals from the PBS injected group could be analyzed.

Statistical analysis

Data were expressed as the means ± SD and differences between means were evaluated using the Student's *t*-test for unpaired data. $p<0.05$ was considered statistically significant.

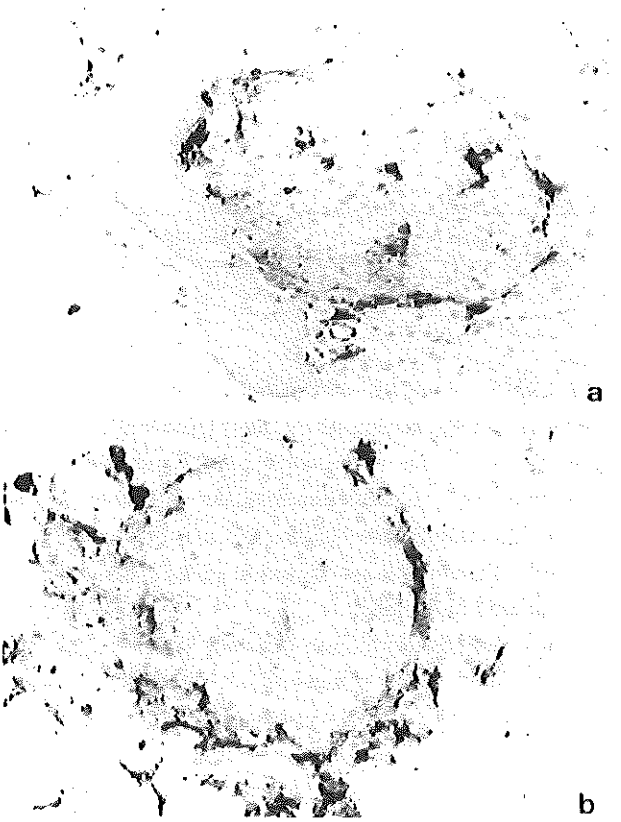

Figure 1 Islet affected by para/peri-insulitis in an 8 weeks old NOD female IT-injected with (a) PBS or (b) syngeneic islet cells. BM8 staining, magn 250x. Note that IT-injection of islet cells prevents BM8 infiltration into the islets, while BM8 is clearly present in the peri-insulitis process. Note also that IT-injection of islet cells does not prevent leucocytic accumulation around the islets. To allow comparison of the islets after treatment, two islets were chosen with a comparable (relatively low) amount of peri-insulitis and without lymphocytic infiltration into the islet.

RESULTS

The presence of lymphocytes as assessed on H+E sections

On histologic examination IT-injection of islet cells significantly increased the percentage of islets affected by para/peri-insulitis ($p = 0.03$; table 1), and significantly reduced the percentage of normal, unaffected islets ($p = 0.02$). Though also the mean percentage of islets affected by an infiltration of lymphocytes tended to be higher, the difference was

not significant (p = 0.07; table 1).

Table 1 Severity of insulitis

experimental group	Insulitis classification % of islets (m±sem)		
	intact	*para/peri-insulitis*	*infiltrative insulitis*
PBS-injected	61.8±23.4	32.8±15.8	5.5±9.7
islet-injected	26.4±12.6	54.6±6.7	19.0±9.5
	p = 0.02	p = 0.03	p = 0.07

BM8 infiltration patterns

Immunohistochemical staining for BM8 revealed that $BM8^+$ macrophages were detectable at three different sites in the spontaneously developing (para/peri-) insulitis of the normal, PBS-injected NOD mouse: a) at the outer edge of the para/peri-insulitis, adjacent to the rim of infiltrated lymphocytes, but not intermingled with these cells; b) in between the lymphocytes forming the para/peri-insulitis; and c) infiltrated into the islet mass itself. For each separate islet in the sections stained for BM8, the presence of $BM8^+$ macrophages at these locations was recorded.

IT injection of islet cells resulted in a significant reduction of islets infiltrated by $BM8^+$ macrophages ($p < 0.0001$, table 2, see figure 1) as compared to PBS-injected controls. It also decreased the percentage of para/peri-insulitis processes in which BM8 could be detected intermingled with the other cells of the insulitis process. This difference, however, was not statistically significant ($p = 0.09$). IT injection of islet cells did not reduce BM8 accumulation at the edge of the para/peri-insulitis process.

Table 2 BM8 infiltration patterns in relation to IT-injection treatment

treatment	BM8 in islets	BM8 in the para/peri-insulitis process	BM8 at the edge of para/peri-insulitis	number of islets scored
IT-islet cells	33±9*	24±4**	71±26	34
IT-PBS	69±6	59±26	93±11	25

* $p<0.0001$ compared to IT-PBS
** $p=0.09$ compared to IT-PBS

DISCUSSION

This study firstly confirms that IT-injection of syngeneic islet cells -despite having favorable effects on ß-cell loss- does not prevent the development of lymphocyte accumulations around the islets. On the contrary, there even was a higher percentage of islets with lymphocytic para/peri-insulitis in the group treated by IT-injection of islet cells. This study is new in that it shows that the composition of the para/peri-insulitis process is altered after IT-injection with islet cells. There clearly was a reduced infiltration by $BM8^+$ macrophages into the islets, an early marker for destructive insulitis, and $BM8^+$ macrophages tended to stay at the outer edge of the para/peri-insulitis infiltrate.

What might be the mechanism by which $BM8^+$ macrophages are not inclined to infiltrate into the islets of NOD-females that have been IT-injected with islet cells? A likely explanation is that $BM8^+$ macrophages need an activation signal, linked to the specific immune system, before being able to infiltrate in the islets, since NOD-*scid/scids* lacking functional B and T cells do not show any infiltration of $BM8^+$ macrophages into the islets. Well known activation signals for macrophages are IFN-γ, TNF-α, and other cytokines that fit into a TH1 cytokine profile[8,9]. A different cytokine profile in the peri-insulitis process, such as a shift in the balance into the direction of Th2 cells or other regulatory T cells might not be able to provide the appropriate signals for the activation of $BM8^+$ macrophages.

This explanation implies that altered cytokine profiles in the para/peri-insulitis process

should be detectable after IT-injection of islet cells. Indeed, mRNA analysis by RT-PCR did reveal a lower message for IFN-γ, TNF-α, TNF-β, and IL-1β in the peri-insulitis of IT-islet-injected NOD females. It, however, also showed a lower message for IL-4 and an elevated message for IL-2 (I.C. Gerling, manuscript in preparation), so a clear-cut shift to a Th2 profile is not likely. Nevertheless, it is clear that cytokine messenger profiles in the para/peri-insulitis process are changed after IT injection of islet cells. The accumulation of diabetes protective T-cells, raised in the thymus by virtue of the IT-islet cell injection might play a role in generating this protective form of para/peri-insulitis.

In conclusion, this report supports the concept that there are at least 2 variants of NOD-para/peri-insulitis. The first type is β-cell destructive, and characterized by an early infiltration of $BM8^+$ macrophages into the islets. The second type is β-cell non-destructive, without infiltration by $BM8^+$ macrophages. More careful studies on the phenotype of leukocytes infiltrating in and around islets are essential in studies on tolerance induction towards ß-cell antigens (IT, BCG, oral immunization) to clarify the role of T cell-macrophage interaction in such protective therapeutic regimens.

REFERENCES

1. Gerling IC, Serreze DV, Christianson SW, Leiter EH. Intrathymic islet cell transplantation reduces β-cell autoimmunity and prevents diabetes in NOD/Lt mice. Diabetes 41:1672-1676,1992
2. Posselt AM, Barker CF, Friedman AL, Naji A. Prevention of autoimmune diabetes in the BB rat by intrathymic islet transplantation at birth. Science 256:1321-1324,1992
3. Jansen A, Homo-Delarche F, Hooijkaas H, Leenen PJ, Dardenne M, Drexhage HA. Immunohistochemical characterization of monocytes-macrophages and dendritic cells involved in the initiation of insulitis and β-cell destruction in NOD-mice. Diabetes 43:667-675,1994
4. Jansen A, Verheul H, Bagchus W, Homo-Delarche F, Drexhage HA. Orchidectomy accelerates the progression of insulitis, focal pancreatitis, and sialoadenitis in male NOD mice. An immunohistopathological study on macrophage and lymphocyte infiltration. Chapter 3.4 of this thesis
5. Jansen A, Homo-Delarche F, Dardenne M, Leiter EH, Drexhage HA. Effect of prophylactic insulin treatment on the number of antigen presenting cells and macrophages in the pancreas of NOD mice. Is the prevention of diabetes based on β-cell rest? Chapter 3.2 of this thesis
6. Prochazka M, Gaskins HR, Schultz LD, Leiter EH. The nonobese diabetic *scid* mouse: a model for spontaneous thymomagenesis associated with immunodeficiency. Proc Natl Acad Sci USA 89:3290-3294,1992
7. Durant S, Coulaud J, Amrani A, Hasnaoui A, Dardenne M, Homo-Delarche F. Effects of various environmental stress paradigms and adrenalectomy on the expression of autoimmune type I diabetes in the non-obese diabetic (NOD) mouse. J Autoimmunity 6:735-751,1993
8. Palmer JP, Helqvist S, Soinas GA, Molvig J, Mandrup-Poulsen T, Andersen H, Nerup J. Interaction of β-cell activity and exposure time in isolated rat islets of Langerhans. Diabetes 38:1211-1216,1989
9. Campbell PA, Canono BP, Cook JL. Mouse macrophages stimulated by recombinant gamma interferon to kill tumor cells are not bactericidal for facultative intracellular bacterium *Listeria monocytogenes*. Immunology 56:1371-1375,1988

Chapter 3.4

ORCHIDECTOMY ACCELERATES THE PROGRESSION OF INSULITIS, FOCAL PANCREATITIS, AND SIALOADENITIS IN MALE NONOBESE DIABETIC (NOD) MICE. AN IMMUNOHISTOPATHOLOGICAL STUDY ON MACROPHAGE AND LYMPHOCYTE INFILTRATION

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SUMMARY

The effect of gonadectomy on the development of insulitis and sialoadenitis in a spontaneous model of type I diabetes, the NOD mouse was studied in two different colonies, by immunohistochemistry, together with its effect on incidence of diabetes.
A panel of monoclonal antibodies, identifying different types of macrophages, dendritic cells, B- and T-lymphocytes, was used to analyze the cellular composition of insulitis and sialoadenitis in NOD mice at various ages (7, 10, 12, and 17 weeks of age). The mice were gonadectomized, non-operated, or sham-operated.
Orchidectomy resulted in an acceleration of the development of both diabetes and sialoadenitis. Ovariectomy had no effect on either disease.
In the pancreases of orchidectomized NODs, there was a faster progression to more severe forms of insulitis and an earlier β-cell loss. There was also an enhancement of the formation of focal leucocytic infiltrates in the exocrine part of the pancreas. In the submandibular gland, a similar faster development of focal leucocytic infiltrates was found in orchidectomized NODs.
Orchidectomy did neither influence the cellular composition of the inflammatory processes (e.g. a predominant infiltration of one specific subclass of macrophages or lymphocytes), nor had it an effect on the order of immune events leading to β-cell destruction, or on the morphology of the islet vasculature.
These observations may be explained by an effect of androgens on the production of cytokines in these inflammatory processes, such as a shift from a Th1 to a Th2 cell response, or a general moderating effect on the production of cytokines, resulting in a slower development of the insulitis and sialoadenitis.

INTRODUCTION

Type I diabetes mellitus is the outcome of a β-cell directed autoimmune process leading to insulitis, β-cell loss and insulin deficiency[1]. In immunohistochemical studies on pancreases of human cases[2,3] and in animal models of the disease[4,5,6,7,8] it has been shown, that the leucocytic infiltrate in and around the islets of type I diabetic subjects is mainly composed of monocyte-derived cells (dendritic cells and macrophages), and of $CD4^+$ and $CD8^+$ T-lymphocytes. *In vivo* studies demonstrated the essential role of both sets of T-cells and macrophages[9,10,11,12]. The most convincing evidence for the role of T-cells of CD4 and CD8 phenotypes consists in transfer experiments establishing that diabetes develops only when both T-cell subsets are transferred[13,14]. Diabetes does also not develop after macrophage elimination, either by the toxic substance silica[10], or by the monoclonal antibody 5C6, which blocks

monocyte-macrophage migration into the tissues[12].

As a further illustration of the important role of monocyte-derived cells in the insulitis process, we recently demonstrated by an immunohistochemical study on NOD mice, that dendritic-shaped cells (MHC class II^+, ER-$MP23^+$) and $MOMA1^+$ macrophages, both thought to be accessory cells in immune responses[8,15,16], are the first cells accumulating around the islets, before any sign of lymphocyte infiltration[8]. It is only after this early accumulation of antigen-presenting cells (APCs) that the classical lymphocytic NOD peri-insulitis develops. During these later phases of lymphocytic para/peri-insulitis an extensive infiltration of the islets by another type of macrophages occurred, i.e. $BM8^+$ macrophages, with phagocytic and cytotoxic capability[17].

Though NOD colonies vary largely in diabetes incidence[18], almost all colonies show an accelerated development of diabetes in females, and the prevalence of diabetes at 30 weeks of age is higher in females than in males[5,18,19,20]. In a previous immunohistochemical study[8], NOD females (Necker's colony, Paris, France) showed a faster progression to more severe patterns of insulitis. A prominent difference between female and male NODs was that $BM8^+$ macrophages infiltrated into the islets as soon as at 10 weeks of age in females, while this BM8 infiltration was absent in males at the age of 17 weeks, the endpoint of our study. If male NODs are gonadectomized the diabetes incidence raises to levels found in females[19,21]. Ovariectomy only has a minor effect on the incidence[19].

In addition to diabetes, NOD mice also develop sialoadenitis, a condition closely resembling Sjögren's syndrome in man[5,18,22]. The mononuclear cell infiltration into the submandibular gland has been reported of mainly consisting of T and B lymphocytes[22]. While most studies investigate the effects of gender on diabetes and insulitis incidence in NOD mice[19,23,24], only one report focussed on its effect on sialoadenitis[25].

In the present study, we addressed the question whether orchidectomy accelerates diabetes development via an effect on the infiltrative behaviour of a single leucocyte population, i.e. an accelerating effect on the influx of $BM8^+$ macrophages into the islets. Since diabetes incidence may vary from one colony to another, we investigated the sequence of the process in two different NOD colonies: the NOD-colony of Organon (Oss, the Netherlands) and the Necker's colony (Hôpital Necker, Paris, France). We additionally analyzed the effect of sex hormones on sialoadenitis and studied the cellular composition of the infiltrates in the submandibular glands.

MATERIALS AND METHODS

NOD colonies and orchidectomy

Eighty male and eighty female NODs from the colony bred at the animal facilities of Organon (Oss, the Netherlands) were included in 2 experimental groups: one group underwent gonadectomy at 4 weeks of age, the other group served as controls (they were not operated). The mice were kept at the Organon-facilities under specific pathogen free conditions.

To confirm the data on the effect of orchidectomy on the insulitis process in the Organon colony, twenty NOD males from the Necker's colony (Hôpital Necker, Paris, France) were included in our immunohistological studies. To exclude the nonspecific effects of the operation itself (e.g. anaesthesia), one half of the Paris NOD-group underwent orchidectomy under "overtin" anesthesia at 4 weeks of age, and the other half was sham-operated. The Necker NOD mice were kept at the Necker facilities under specific pathogen free conditions as described previously.

Two male and two female BALB/c mice of 10 weeks of age were used as controls for staining the cells infiltrating the islets and the matrix of the islet vessels.

Diabetes incidences

In both colonies, diabetes development was monitored weekly (Diabur test 5000 or Glukotest, Boehringer-Mannheim, Mannheim, Germany). In the Organon colony, diabetes onset was determined in all mice for the immunohistological study. In Necker's colony, two different groups of mice were investigated: one for the immunohistological study (mice were sacrified at 12 weeks of age) and one for an incidence study (follow-up until 30 weeks of age). The log-rank and the chi-square test (Life test and frequency procedures in SAS, version 6 addition, SAS Institute Inc, 1990) were used to analyze differences between the distribution curves and the prevalence of diabetes at 23 and 30 weeks of age for the Organon and Necker's colony respectively, between the gonadectomized group and its respective control. According to the colony, 10 to 15 animals were included in each group.

preparation of the pancreases and submandibular glands

At the age of 7, 10, and 17 weeks, 10 mice of each experimental group in the Organon facilities were killed by cervical dislocation after bleeding from the orbital sinus under ether anaesthesia. Mice were routinely checked for succes of orchidectomy. Pancreas and one submandibular gland from each animal were removed, embedded in Tissue-tek and frozen in liquid nitrogen chilled isopentane for immunohistochemical studies. The other submandibular gland was fixed and embedded in paraffin according to standard

methods to assess the severity of sialoadenitis.[26] Tissues were stored at -80°C untill immunohistochemistry was performed.
Mice from the Necker's colony were killed at 12 weeks of age, by the same procedure. Only pancreatic material was removed. Tissue preparation was identical to that described above.

assessment of severity of sialoadenitis
Three sections (5 μm) of each paraffin embedded submandibular gland were cut, mounted and stained with haematoxyline-eosine (H+E staining) according to standard methods. Mononuclear focal infiltrates were scored independently by two observers on a semi-quantitative scale of 0-5, taking into account the number and the size of the infiltrates. The score per animal and per treatment group was calculated and statistically evaluated with the Wilcoxon test.

Table 1 Monoclonal antibodies used for this immunohistochemical study

monoclonal antibody	specificity	source
F4/80	mature macrophages	ATCC
M1/70	mac-1, CR3	ATCC
BM8	macrophages with phagocytosing capacity	BMA
ER-MP23	38 kD polypeptide of unknown function, present on subset of APCs	P.Leenen
MOMA-1	marginal metallophylic macrophages	G.Kraal
KT3	CD3	ATCC
RA3.6B2	B220 antigen	ATCC
30G12	CD45	ATCC
B220	B220 antigen	Pharmingen, USA
α-TCRαβ	TCRαβ	Pharmingen, USA
α-CD4	CD4	Pharmingen, USA
α-CD8	CD8	Pharmingen, USA
78B33	laminin	A. Sonnenberg

monoclonal antibodies used for immunohistochemistry

Table 1 shows the monoclonal antibodies used to identify different types of macrophages and dendritic cells, the lymphocytes, and the extra-cellular matrix component laminin in the pancreases and the submandibular glands. F4/80[27], M1/70[28], ER-MP23[29], MOMA1[15], KT3, RA3.6B2, and 30G12 were rat-anti-mouse hybridoma culture supernatants obtained from our department and used undiluted. BM8[17] was obtained from BMA (Augst, Switzerland) and was used diluted 1/25 in PBS with 1% BSA. These markers were detected with horseradish peroxidase (HRP) labeled rabbit-anti-rat immunoglobulins (DAKO, Glostrup, Denmark). For the staining of lymphocyte populations in the submandibular glands B220, α-TCRαβ, αCD-4, and αCD-8 (Pharmingen, USA) were used in dilutions 1/50 or 1/100.

Guinea-pig anti-insulin polyclonal antibody (DAKO) was used diluted 1:175 in PBS with 1% BSA, and detected with HRP labeled rabbit-anti-guinea pig immunoglobulins (DAKO). Rabbit-anti-glucagon polyclonal antibody (DAKO) was diluted 1:75 in PBS with 1% BSA and detected with HRP labeled swine-anti rabbit immunoglobulins (DAKO).

78B33[26], a culture supernatant, was kindly donated by dr A. Sonnenberg, and used in a dilution of 1:15 in PBS with 1% BSA.

immunohistochemical procedure

Immunohistochemistry was largely performed as described recently[8]. Briefly, the following procedure was carried out: before sectioning, microscopic slides were treated by a 10 min exposure to 95% ethanol/5% diethylether, dried in air, coated with a solution of 0.1% gelatin/0.01% chromiumalum in distilled water, and dried in air again. Thereafter, 5 μm cryo-sections of the stored tissue specimens were cut, dried in air overnight, and fixed by either pararosaniline fixation (ER-MP23) or acetone fixation (all other monoclonal and polyclonal antibodies). Before exposure to the first step of monoclonal and polyclonal antibodies, the tissues were preincubated for 10 minutes with 4% (v/v) normal rabbit serum. In case of the glucagon staining, this preincubation was carried out with 4% (v/v) normal swine serum. Without washing first step monoclonal and polyclonal antibodies were applied and incubated for 1 hr at room temperature. Thereafter, sections were washed three times in PBS with 0.1% BSA for a total of 15 minutes. To diminish background staining 1% of normal mouse serum was added to the HRP-labeled second step antibodies diluted in PBS with 1% BSA. This mixture was allowed to react for 10 minutes and was then applied to the cryosections. After 30 minutes of incubation at room temperature sections were washed three times in PBS. Thereafter, the HRP label was developed for 4 minutes by 0.05% DAB/0.02%H2O2 dissolved in PBS. Excess of DAB was removed by streaming tap water. After that a short counterstaining with hematoxilin was performed for

histomorphologic purposes. The slides were dehydrated by an ethanol/xylene series and covered with Depex.

staging of islet inflammation based on the pattern of macrophage/lymphocyte infiltration
All islets in every section-series made from each individual NOD pancreas were classified according to the staging criteria as described recently[8]. (See this reference for a more detailed description and extensive photography.) Briefly these stages can be described as follows:

stage 0:	Intact islet as observed in control BALB/c mice. This means a few ER-MP23$^+$ dendritic cells and MOMA1$^+$ macrophages at the edge of the islet, but no more than 2-3 per islet section.
stage I:	Intact islet, however blood vessels near the islet display a high endothelium and an enlarged perivascular area. There is an accumulation of a few ER-MP23$^+$ dendritic cells and MOMA1$^+$ macrophages in this perivascular area.
stage II:	Abnormal vessels as in stage I, with islet now surrounded by a raised number (more than 3 per islet section) of ER-MP23$^+$ dendritic cells and MOMA1$^+$ macrophages. A few BM8$^+$ macrophages at the edge of the islet. Lymphocytes are still not present around and in the islets.
stage IIb:	As stage II, but BM8$^+$macrophages can be detected that have infiltrated into the islet itself
stage III:	As stage II, but with lymphocytes present at one pole of the islet (para-insulitis)
stage IIIb:	As stage III, but BM8$^+$macrophages can be detected that have infiltrated into the islet
stage IV:	As stage III, with lymphocytes now surrounding the whole of the islet, but not infiltrating it (peri-insulitis)
stage IVb:	As stage IV, but BM8$^+$macrophages can be detected that have infiltrated into the islet
stage V:	Both lymphocytes and ER-MP23$^+$ and BM8$^+$macrophages show massive infiltration into the islet. Insulin-containing cells are still present
stage VI:	Insulin-containing cells are no longer detectable (endstage)

For each NOD pancreas the percentage of islets in a given stage was computed. For each experimental group the mean percentage of islets in a given stage was determined

by adding up the staging percentages of each individual pancreas and dividing it by the number of animals in that group (usually 10). In this way, all mice weighed equal in the description of the profile for the whole of the experimental group. In the groups of 7-12 weeks of age the percentage of islets infiltrated by $BM8^+$ macrophages was computed by adding up the individual percentages in each mouse and dividing it by the number of mice in the group.

quantification of surface area in the section of focal leucocytic infiltrates in the exocrine pancreas

Focal infiltrates of leucocytes could also be detected in the exocrine pancreas of some NODs (particularly in the Organon colony). The total surface area occupied by the focal infiltrates in the plane of the section in the exocrine pancreas, as well as the total surface area of the pancreas section in which the focal infiltrates were found were assessed via a VIDAS-RT image analysis system (Kontron Elektronik GmbH/ Carl Zeiss, Weesp, the Netherlands). In this way it was possible to calculate the extent of leucocyte infiltration in the exocrine pancreas. The extent of focal leucocytic infiltration in the exocrine pancreas was expressed as the surface area in μm^2 occupied by focal infiltrates per 1000 μm^2 surface area of exocrine pancreas tissue. Statistical comparison of the extent of leucocytic infiltration in the exocrine pancreas between groups was carried out by the two-sample, two-tailed Mann-Whitney signed rank test. Comparison of the frequency of focal infiltrates observed in the exocrine pancreas was performed with the two-tailed Fisher's exact test.

RESULTS

Incidence of diabetes and severity of sialoadenitis in gonadectomized and non-gonadectomized mice

Orchidectomy at 4 weeks of age in NOD males from both colonies resulted in similar results. Significant accelerated diabetes onset was observed in the Organon ($p = 0.0008$) and Necker's colony ($p < 0.0001$) (fig. 1). Moreover, there was a significant increased prevalence (x 4 fold) at 23 weeks of age in the Organon and, at 30 weeks of age in the Necker's colony ($P = 0.002$ and $P = 0.0018$, respectively) (fig. 1).

With regard to sialoadenitis, focal leucocytic infiltrates in between the exocrine acini could easily be detected in H+E stainings. The sialoadenitis was more severe in intact females than in the intact (non-orchidectomized) males already at week 7 and this phenomenon persisted until week 17 (table 2). Ovariectomy did not have any effect on the severity and the progression of sialoadenitis, while orchidectomy clearly accelerated the development of sialoadenitis and increased its severity.

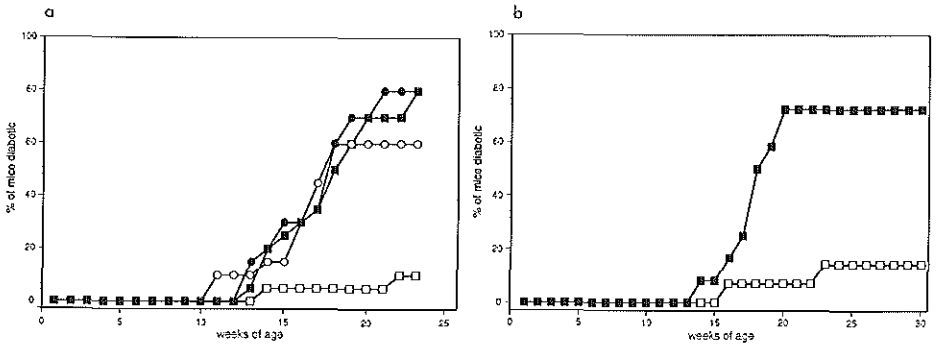

Figure 1a Diabetes development in gonadectomized and non-gonadectomized male and female NODs from the Organon-colony [orchidectomized males ■, non-orchidectomized males □, ovariectomized females ●, non-ovariectomized females ○]. Note that orchidectomy results in a rise in male diabetes development to levels found in females. Note also that ovariectomy had no significant effect in this experiment.

Figure 1b Diabetes development in orchidectomized (▩) and sham-operated (▫) NOD-males from the Necker's Paris-colony. Note that orchidectomy shows similar effects on diabetes development as in the Organon colony.

Since ovariectomy had no significant effect on diabetes incidence and the incidence and severity of focal leucocytic infiltrates in the submandibular gland, immunohistochemically detectable differences in the composition of the insulitis and sialoadenitis in ovariectomized and non-ovariectomized animals were not to be expected. Therefore, we decided to focus on the effect of orchidectomy on immunohistochemical changes with regard to the insulitis and sialoadenitis process.

Table 2 Effects of gender and gonadectomy on the severity of sialoadenitis

gender	*gonadectomy*	*sialoadenitis score (±SEM) at week*		
		7	10	17
male	no	0.2±0.2	0.1±0.1	0.9±0.2
male	yes	0.2±0.1	1.4±0.2*	2.1±0.2*
female	no	1.4±0.3*	2.2±0.2*	2.8±0.4*
female	yes	1.5±o.4	3.0±0.2	3.2±0.2

*: $p < 0.05$ (Wicoxon Test) compared to intact males

The macrophage-lymphocyte infiltration around and into the islets of orchidectomized vs non-operated/sham-operated NOD males

We were not able to detect an enhancing effect of orchidectomy on the infiltrative capacity of one given leucocytic population, all leucocytic populations were equally

affected. There was only a faster progression of the insulitis process as a whole (see fig 2a+b):

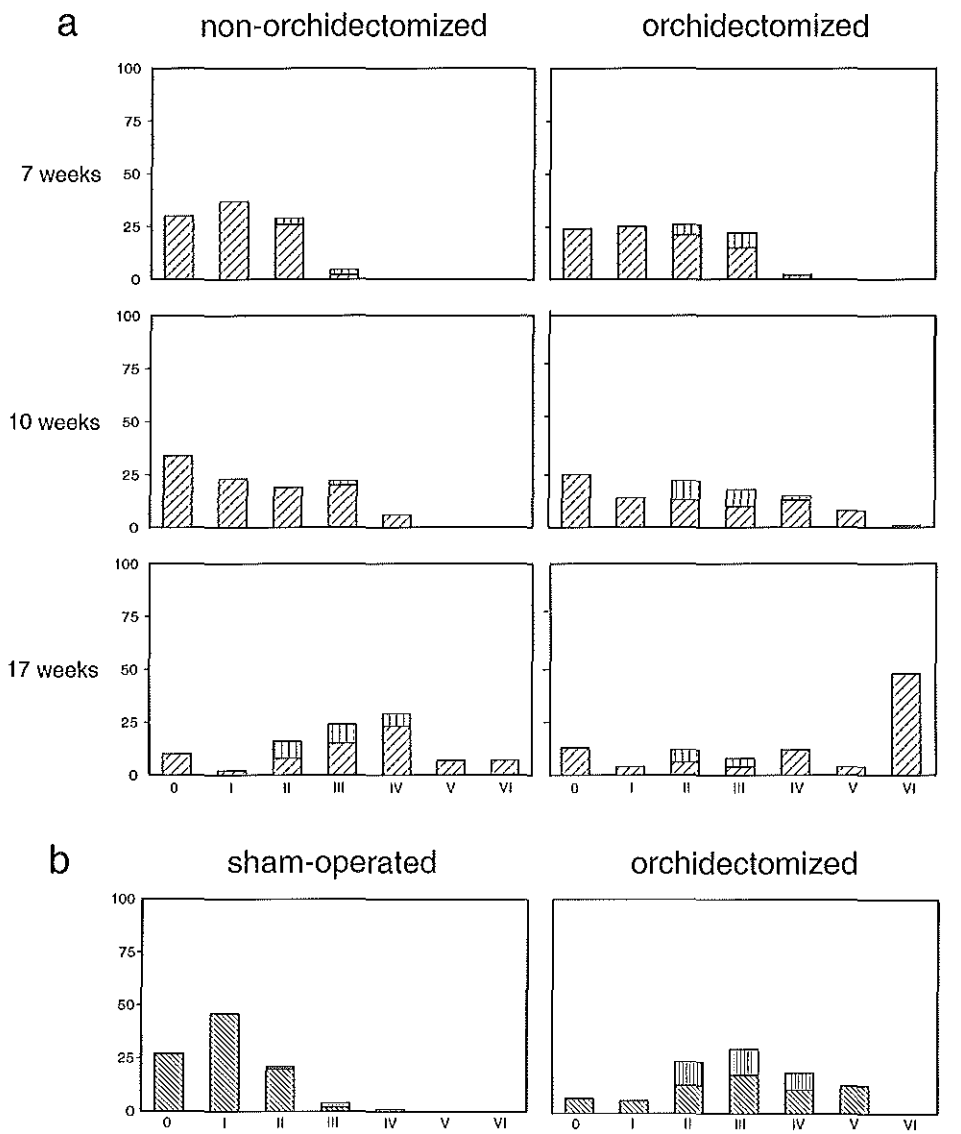

Figure 2. Distribution (%) of type/severity of islet infiltration in NOD males according to age and experimental condition. See materials and methods for staging criteria and distribution calculation procedure. Each treatment-age group consisted of 8-10 animals. Vertical hatching indicates percentages of islets with infiltration by $BM8^+$ macrophages *into* the islets. a) distribution in orchidectomized and non-operated NOD males of 7, 10, and 17 weeks of age from Organon-colony. b) distribution in 12- week-old orchidectomized and sham-operated NOD-males from the Necker's-colony.

Firstly: With regard to APC accumulation at the circumference of the islet (islet stage II or more progressed) 50% of islets had reached these stages in orchidectomized NODs at 7 weeks of age, whereas the non-operated controls only showed 34% of the islets in these stages (difference statistically significant, $X^2 = 5.93$, $p = 0.015$).
Secondly: With respect to lymphocytic infiltration around or into the islet (stage III or higher) 24% of the islets in orchidectomized NODs had reached this stage at 7 weeks of age, as compared to 4.5% in non-operated animals ($X^2 = 13.1$, $p = 0.0002$). At 10 weeks of age the difference in lymphocyte involvement was still present: 42% of the islets had reached stage III or higher in the orchidectomized group, as compared to 28% in the non-orchidectomized animals ($X^2 = 3.8$, $p = 0.05$).
Thirdly: Considering infiltration by $BM8^+$ macrophages in the early stages of para/peri-insulitis there was a substantial infiltration by $BM8^+$ macrophages *into* the islets already at relatively young age in orchidectomized males: at 10 weeks of age 19% of the islets was infiltrated by BM8, compared to 2% in non-orchidectomized animals ($X^2 = 13.7$, $p = 0.0002$; table 3)
Fourthly: With regard to β-cell loss: at 17 weeks of age almost half of the islets (48%) found in the orchidectomized group were negative for insulin, and thus defined to be endstage islets (stage VI), as compared to 7% in the non-orchidectomized group (fig 2a; $X^2 = 40.1$, $p < 0.00001$)) .

Table 3 Percentage of islets infiltrated by $BM8^+$ macrophages, as related to age and treatment

age/colony	treatment	
	non-orchidectomized or sham-operated	*orchidectomized*
7 weeks/Organon	5.5	11
10 weeks/Organon	2	19
12 weeks/Necker	1	31

The data in the orchidectomized compared to the sham-operated experimental animals from the Necker-colony confirmed the results of the Organon colony: at 12 weeks of age, 62% of the islets were surrounded or infiltrated by lymphocytes (stage III or higher) in the orchidectomized group, with 31% of the islets infiltrated by $BM8^+$ macrophages, whereas in the sham-operated group only 5% were in these advanced stages, and only 3% was infiltrated by BM8 (fig 2b; $X^2 = 70.4$, $p < 0.00001$ and $X^2 = 31.3$, $p < 0.00001$ respectively).

In gonadectomized as well as in non-operated or sham-operated males from both colonies, the insulitis process started with a more prominent appearance of the connective tissue area surrounding the blood vessels located near the islet, as previously described in intact NOD females[8]. The endothelium of these vessels was swollen. In this study, histological differences in neither the endothelium of the vessels close to the islets nor the perivascular area itself could be detected between orchidectomized and control animals. An immunohistochemical staining for laminin in the peri-vascular area of the islet vessels, confirmed the enlargement of this area in NODs compared to control BALB/c mice (fig. 3a and b), but did not reveal differences between orchidectomized and non-orchidectomized NODs.

The immunohistochemistry of focal leucocytic infiltrates in the exocrine pancreas and the submandibular gland.

Besides the para- and peri-insular infiltration of leucocytes, focal accumulations of such cells could also be detected scattered throughout the exocrine pancreas and spreading in between the exocrine acini. These focal leucocytic infiltrates were primarily localized in connection to vessels in the pancreas. Insulin$^+$- or glucagon$^+$-cells could not be detected in these focal infiltrates. The exocrine inflammatory foci were particularly evident in the Organon colony, but also a few small infiltrates were found in 2 mice of the Necker's colony. They were histologically very similar to the focal leucocytic infiltrates in the submandibular glands. The pancreatic focal infiltrates were mainly composed of T cells and B cells. Especially at later age (17 weeks) and in the larger infiltrates the B-cell component was prominently present, but a specific localization was not apparent. F4/80$^+$ and BM8$^+$ macrophages were found intermingled with the lymphocytes, but clearly stained stronger at the periphery of the infiltrates. This pattern was also seen for MOMA1, but the number of MOMA1$^+$ cells was considerably lower than the number of F4/80$^+$ and BM8$^+$ macrophages. ER-MP23$^+$ cells were intermingled with lymphocytes, and did not show any preference in location in the infiltrate. In addition to the focal infiltrates, there was a scattered infiltration of ER-MP23$^+$, MOMA1$^+$, F4/80$^+$, and BM8$^+$ macrophages in the connective tissue septa of the entire gland.

Our data did not indicate a difference in the composition of focal infiltrates of orchidectomized as compared to non-orchidectomized NODs, but the focal leucocytic infiltrates in the exocrine pancreas developed faster in females and in orchidectomized animals as compared to control male NOD-mice. At 10 weeks of age, these infiltrates occupied significantly more surface area in orchidectomized vs non-orchidectomized animals ($p = 0.02$, table 4).

Figure 3. Laminin staining of vessels in the pancreas of (a) a 10-week-old non-orchidectomized male NOD (magn 260x) and (b) a 7-week-old control male BALB/c mouse (magn 250x). Note the enlarged peri-vascular area and the high endothelium (arrow) in the vessels of the NOD pancreas.

A limited number (n = 12) of frozen submandibular glands from gonadectomized and non-gonadectomized males and females animals, having a different severity of sialoadenitis scores were immunohistochemically evaluated (fig. 4).

B-cells ($B220^+$ cells) were clearly found at the outer parts of the infiltrates, whereas T cells ($TCR\alpha\beta$, CD4, or CD8 positive cells) were mainly found in the centre of the infiltrates. Again, $BM8^+$ and $F4/80^+$ cells were localized throughout the whole gland, concentrated at the edge of the acini. Small numbers of $BM8^+$ cells were present in the focal infiltrates with a preference to the periphery of the infiltrates. Also $F4/80^+$ cells seemed to concentrate at the periphery of the infiltrates. Low numbers of $MOMA1^+$

cells were localized in the connective tissue of the glands and in the infiltrates. In a few glands they were not detectable. Our data did not indicate a difference between intact males and females or a specific effect of gonadectomy with respect to the composition and localization of the cells. They did however develop earlier in females and gonadectomized males.

Table 4

Infiltrates found in the exocrine pancreas in orchidectomized (OCX) vs non-orchidectomized (non-OCX) NOD males from the Organon colony

group	number of animals with infiltrates	p-value (Fisher's exact test)	Inf (μm^2)/1000μm^2 of pancreas (mean ± sd)	p-value (ranked sum test)
♂ 7w non-OCX	3/10	0.18	4.7 ± 10.7	0.41
♂ 7w OCX	7/10		5.7 ± 9.7	
♂ 10w non-OCX	5/8	0.20	4.5 ± 9.2	0.02
♂ 10w OCX	8/8		22.4 ± 19.3	
♂ 17w non-OCX	9/10	1.0	17.2 ± 15.6	0.97
♂ 17w OCX	10/10		18.4 ± 14.8	

DISCUSSION

The present study firstly confirms earlier reports on the effect of gonadectomy on diabetes incidence in NOD-mice[19,21]: Ovariectomy of NOD females had virtually no effect; orchidectomy resulted in a higher diabetes prevalence in NOD males, comparable to that seen in females of the same colony. Androgen treatment of NOD females, either started neonatally or after the onset of insulitis is known to decrease the incidence of diabetes[27,28]. This indicates that androgens probably control the progression of the disease, exerting a protective effect on spontaneous diabetes development in NOD males.

In addition, our study also shows that sialoadenitis in the NOD mice is subject to hormonal control: females were more severely afflicted than males and castration of males aggravated sialoadenitis. Again, ovariectomy had no effect. These results are in contrast with one previous report on sialoadenitis in the NOD mouse[25]. However, our

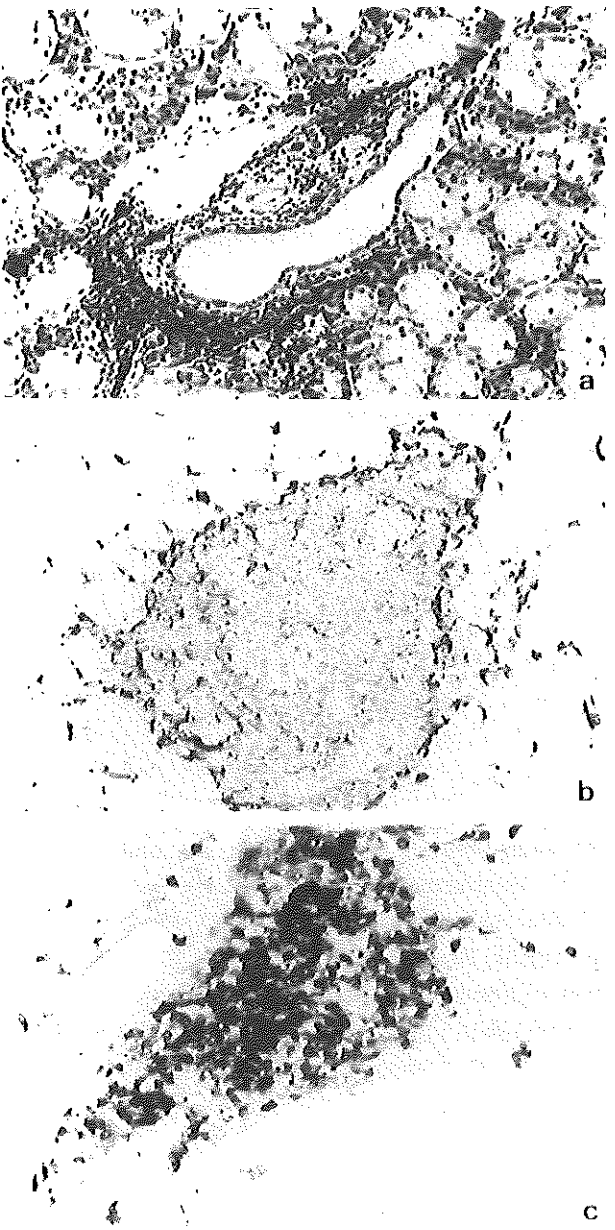

Figure 4a. Infiltrate in the submandibular gland of a 17-week-old orchidectomized male NOD mouse. H+E staining, magn 160x.

Figure 4b.+c. Infiltrate in the exocrine pancreas of a 17-week-old gonadectomized male NOD mouse. F4/80 staining (b), B220 staining (c), magn. 160x. Note that macrophages are primarily present at the outer edges of the infiltrate. Note also the predominant presence of B-cells.

results comply with the observations with respect to gender and gonadectomy in another model of sialoadenitis, the NZB/W mouse[29,30]. This is further supported by the beneficial effects of androgens[31], and by the lack of effects of ethinyl oestradiol on sialoadentis in the NZB/W mice[32].

With respect to the cellular composition of the focal infiltrates in the submandibular glands and the localization of the various mononuclear cell markers, our results confirm the previous observations[22] in that the B cells are localized at the periphery, whereas the T cells are at the centre of the infiltrates. In addition, we did not find a specific localization of the $CD4^+$ or $CD8^+$ cells in the T cell areas. Cells positive for a given macrophage markers did not seem to be preferentially localized in the infiltrates, but were prevalent throughout the gland.

Our data further indicate that the compositions of the focal infiltrates both in the submandibular gland as well as in the endocrine pancreas are not affected by hormonal manipulation. Also the composition of the different sequential stages of the insulitis process in the pancreas was not altered by orchidectomy. Neither in the Organon-colony, nor in the Necker colony differences in the composition and the sequence of development of the peri-insulitis process in androgen-deprived vs. non-androgen-deprived NOD males were found. Therefore, there was no histological evidence for an exclusive stimulation or downregulation of a given leucocyte population, such as a higher activation state of phagocytic/cytotoxic $BM8^+$ macrophages, resulting in higher numbers of such macrophages in the islets, or an infiltration into the islets by $BM8^+$ macrophages at earlier stages of para/peri-insulitis. There was, in fact a general enhancement of the development of the infiltrative processes, affecting all macrophage and lymphocyte populations. Our histopathological data, however, do not exclude the possibility that androgen-deprivation specifically and exclusively stimulates the function of the first infiltrating antigen-presenting cells, which would also result in a faster progression of the autoinflammatory response in general.

The focal leucocytic infiltrates in the exocrine pancreas of the orchidectomized NOD males closely resemble the infiltrates described by us before in the pancreas of an 8 months old child, who died from a rapid developing form of diabetes[3]. Like the focal infiltrates in orchidectomized NOD males, the focal infiltrates in the pancreas of this diabetic child were composed of a low-organized mixture of T-cells, B-cells, dendritic cells, and macrophages. In these pancreatic infiltrates B cells were not as prominently present as in the NOD pancreases. In this diabetic child, the infiltrates spreaded throughout the pancreas in between the exocrine acini, and were not limited to the

major connective tissue septa. We then interpreted these larger focal leucocytic infiltrates in the exocrine pancreas as closely linked to the insulitis process, and since these focal infiltrates also occurred in normal fetal pancreases, we discussed their function as possibly related to tolerance induction to islet antigens. The present data however, showing a striking similarity between the focal infiltrates in the exocrine pancreas and the submandibular gland of the NOD mouse may suggest that the focal infiltrates in the exocrine pancreas represent a separate autoimmune process directed towards exocrine glandular tissue. It remains therefore puzzling why such infiltrates occur in normal fetal and neonatal pancreases, how and if they are related to the insulitis process and whether their presence indeed indicates a more severe form of insulitis.

What is presently known about the effects of androgen deprivation on immune responsiveness? A sexual dimorphism of the immune response has already been recognized for a long time (reviewed in[33,34]): females in general have a stronger immune response both primary and secondary as compared to males. Females also have a higher thymus weight, and higher serum immunoglobulin concentrations. Moreover, females are more resistant to the induction of immunological tolerance and have a greater ability to reject tumors and allografts. The mode of action of sex steroids in spontaneous autoimmune models remains to be elucidated. However, amelioration of autoimmune symptoms by particularly androgens have been observed[30], and it is conceivable that these hormones may affect the differentiation of immunocompetent cells. Several observations in the literature support this hypothesis: various reports indicate that sex hormones may interfere in different ways with cytokine production[35], for example IFNγ[36], IL-1[37], IL-2[38], IL-5[39],and TGFβ[40], although the results are sometimes inconclusive.
There are indications that Th1 cells play an important role in the development of cell-mediated autoimmune diseases[41], such as in type I diabetes. Also in the NOD mice Th1 cells play an important role, since disease-transferring T cells produce IFNγ[42] and treatment with anti-IFNγ reduced disease[43]. Furthermore, the administration of IL-4 or IL-10 (stimulating Th2) favourably affects disease in NOD mice[44,45]. Whether the shift of Th1 to Th2 cells, or a general suppression by androgens of cytokine production from lymphocytes and macrophages play a role in our model of gonadectomy remains to be investigated.

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REFERENCES

1. Powers, AC, Eisenbarth GS. Autoimmunity to islet cells in diabetes melitus. Ann Rev Med 1987;36:533-544.
2. Foulis AK, Liddle CN, Farquharson MA, Richmond JA, Weir RS. The histopathology of the pancreas in type I (insulin dependent) diabetes mellitus: a 25 year review of deaths in patients under 20 years of age in the United Kingdom. Diabetologia 1986;29:267-274.
3. Jansen A, Voorbij HAM, Jeucken PHM, Bruining GJ, Hooijkaas H, Drexhage HA. An immunohistochemical study on organized lymphoid cell infiltrates in fetal and neonatal pancreases. A comparison with similar infiltrates found in the pancreas of a diabetic infant. Autoimmunity 1993;15:31-38.
4. Voorbij HAM, Jeucken PHM, Kabel PJ, de Haan M, Drexhage HA. Dendritic cells and scavenger macrophages in pancreatic islets in prediabetic BB rats. Diabetes 1989;38:1623-1629.
5. Makino S, Kunimoto K, Muraoka Y, Mizushima Y, Katagiri K, Tochino Y. Breeding of a non-obese diabetic strain of mice. Exp Anim 1980;29:1-13.
6. Miyazaki A, Hanafusa T, Yamada K, Miyagawa J, Fujino-Kurihara H, Nakajima H, Nonaka K, Tarui S. Predominance of T-lymphocytes in pancreatic islets and spleen of pre-diabetic non-obese diabetic (NOD) mice: a longitudinal study. Clin Exp Immunol 1985;60:622-630.
7. Reddy S, Lui W, Elliott RB. Distribution of pancreatic macrophages preceding and during early insulitis in young NOD mice. Pancreas 1993;8:602-608.
8. Jansen A, Homo-Delarche F, Hooijkaas H, Leenen PJ, Dardenne M, Drexhage HA. Immunohistochemical characterization of monocytes-macrophages and dendritic cells involved in the initiation of the insulitis and β-cell destruction in NOD-mice. Diabetes 1994;43:667-675.
9. Le KU, Amano K, Yoon JW,. Evidence for initial involvement of macrophages in development of insulitis in NOD-mice. Diabetes 1988;37:989-991.
10. Charlton B, Bacelj JA, Mandel TE. Administration of silica particles or anti-Lyt-2 antibody prevents β-cell destruction in NOD mice given cyclophosphamide. Diabetes 1988;37:930-935.
11. Ihm SH, Yoon JW. Studies on autoimmunity for initiation of β-cell destruction. VI Macrophages essential for development of β-cell specific cytotoxic effects and insulitis in NOD-mice. Daibetes 1990;39:1273-1278.
12. Hutchings P, Rosen H, O'Reilly L, Simpson E, Gordon S, Cooke AV. Transfer of diabetes in mice prevented by blockade of adhesion-promoting receptor on macrophages. Nature 1990;348:639-642.
13. Miller BJ, Appel MC, O'Niel JJ, Wicker LS. Both the Lyt2$^+$ and the L3T4 T cell subsets are required for the transfer of diabete in NOD mice. J Immunol 1988;140:52-58.
14. Christianson SW, Shultz LD, Leiter EH. Adoptive transfer of diabetes into immunodeficient NOD-scid/scid mice. Relative contributions of CD4$^+$ and CD8$^+$ T-cells from diabetic versus prediabetic NOD.NON-Thy-1a donors. Diabetes 1993;42:44-55.
15. Kraal G, Janse M. Marginal metallophylic macrophages of the mouse spleen identified by a monoclonal antibody. Immunology 1986;58:665-669.
16. Kraal G, Janse M, Claassen E. Marginal metallophylic macrophages in the mouse spleen: effects of neonatal injections of MOMA-1 antibody on the humoral immune response. Imm Lett 1988;17:139-144.
17. Mallorny U, Michels E, Sorg C. A monoclonal antibody against an antigen present on mouse macrophages and absent from monocytes. Cell Tissue Res 1986;243:421-428.
18. Pozzilli P, Signore A, Williams AJK, Beales PE. Nod colonies around the world - recent facts and figures. Immunolgy Today, 1993;14:193-196.
19. Fitzpatrick F, Lepault F, Homo-delarche F, Bach JF, Dardenne M. Influence of castration, alone or combined with thymectomy, on the development of diabetes in the nonobese diabetic mouse. Endocrinology 1991;129:1382-1390.

20. Bach JF. Insulin-dependent diabetes mellitus as an autoimmune disease. Endocrine Reviews 1994;15:1-27.
21. Makino S, Kunmoto K, Muraoka Y, Katagiri K. Effect of castration on the appearance of diabetes in NOD mouse, Exp Anim 1981;30:137-140.
22. Miyakawa J, Hanafusa P, Miyazaki A, Yamada K, Fujino-Kurihara H, Nakajima H, Kona N, Nonaka K, Tochino Y, Tarui S. Ultrastructural and immunocytochemical aspects of lymphocytic submandibulitis in the non-obese diabetic (NOD) mouse. Virchows Arch 1986;51:215-225.
23. Kanazawa Y, Tochino Y, Komeda K. Non-obese diabetic mice: solved and unsolved problems of pathogenesis. In: the Immunology of Diabetes Mellitus. Eds Jaworski et al, 1986;pp113-119.
24. Leiter EH, Serreze DV, Prochazka M. The genetics and epidemiology of diabetes in NOD mice. Immunology Today 1990;11:147-149.
25. Goillot E, Mutin M, Louraine J-L. Sialoadenitis in Nonobese diabetic mice: Transfer into syngeneic healthy neonates by splenic T lymphocytes. Clin Immunol Immunopathol 1991;59:462-473.
26. Bosman FT, Lindeman J, Kuiper G, Walt A van der, Krenning J. The influence of fixation on the immunoperoxidase staining of plasma cells in paraffin sections of intestinal biopsy specimens. Histochemistry 1977;153:57-62.
27. Austyn JM, Gordon S. F4/80, a monoclonal antibody dircted specifically against the mouse macrophage. Eur J Immunol 1981;11:805-815.
28. Springer TA, Galfre G, Secher DS, Milstein C. Mac-1: a macrophage differentiation antigen identified by monoclonal antibody. Eur J Immunol 1979;9:301-306.
29. Leenen PJM, Melis M, Slieker WAT, Van Ewijk W. Do ER-MP23$^+$connective tissue macrophages constitute a separate lineage of macrophage differentiation? J Leucocyte Biol 1991;2(suppl):63-64.
26. Sonnenberg A, Daams H, van der Valk M, Hilkens J, Hilgers J. Development of mouse mammary gland: Identification of stages in differentiation of luminal and myoepethelial cells using monoclonal antibodies and polyvalent antiserum against keratin. J Histochem Cytochem 1986;34:1037-1046.
27. Fox HS. Androgen treatment prevents diabetes in nonobese diabetic mice. J Exp Med 175:1409-1412,1992
28. Hawkins T, Gala RR, Dunbar JC. The effect of neonatal sex hormone manipulation on the incidence of diabetes in nonobese diabetic mice. Proc Soc Exp Biol Med 202:201-205,1993
29. Verheul HAM, Schot PC, Deckers GHJ, Schuurs AHWM. Effects of tibolone, lynestrenol, ethylestrenol and desogestrel on autoimmune disorders in NZB/W mice. Clin Immunol Immunopathol 38:198-208,1986
30. Schuurs AHWM, Verheul HAM. Effects of gender and sex steroids on the immune response. J Steroid Biochem 1990;35:157-172.
31. Schot LPC, Verheul HAM, Schuurs AHWM. Effect of nandrolone decanoate on Sjögren's syndrome-like disorders in the NZB/W mice. Clin Exp Immunol 1984;57:571-574.
32. Verheul HAM, Verveld M, Hoefakker S, Schuurs AHWM. Effects of ethinylestradiol on the course of spontaneous autoimmune disease in NZB/W and NOD mice. Immunopharmacol Immunotoxicol submitted.
33. Grossman CJ. Possible underlying mechanisms of sexual dimorphism in the immune response, fact and hypothesis. J Steroid Biochem 1989;34:241-251.
34. Homo-Delarche F, Fitzpatrick F, Christeff N, Nunez EA, Bach JF, Dardenne M. Sex steroids, glucocorticoids, stress and autoimmunity. J Steroid Biochem Molec Biol 1991;40:619-637.
35. Wegmann TG, Lin H, Guilbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a Th2 phenomenon?. Immunology Today 1993;14:353-356.
36. Sarvetnick N, Fox HS. Interferon gamma and the sexual dimorphism of autoimmunity. Med Biol Med 1990;7:323-331.
37. Polan ML, Daniele A, Kuo A. Gonadal steroids modulate human monocyte IL1 activity. Fert and Ster 1988;49:961-968.
38. Daynes RA, Araneo BA, Dowell TA, Huang K, Dudley D. Regulation of murine lymphokine production in vivo:II. Dehydro-epiandrosterone is a natural enhancer of interleukin-2 synthesis by T helper cells. Eur J Immunol 1990;26:793-802.
39. Wang Y, Campbell HD, Young IG. Sex hormones and dexamethasone modulate interleukin 5 gene expression in T lymphocytes. J Steroid Biochem 1993;44:203.
40. Olsen NJ, Zhou P, Ong H, Kovacs WJ. Testosterone induces expression of transforming growth factor-beta in the murine thymus. J Steroid Biochem Molec Biol 1993;45:327-332.
41. Romagnani S. Lymphokine production by human T cells in disease states. Annu Rev Immunol 1994;12p:227.

42. Shimizu J, Kanagawa O, Unanue ER. Presentation of beta-cell antigens to $CD4^+$ and $CD8^+$ T cells of non-obese diabetic mice. J Immunol 1993;151:1723-1739.
43. Campbell IL, Kay TWH, Oxbrow L, Harrison LC. Essential role for interferon gamma and interleukin 6 in autoimmune insulin dependent diabetes in NOD/Wehi mice. J Clin Invest 1991;87:739-742.
44. Rapoport ML, Jaramillo A, Zipris D, Lazarus AH, Serreze DV, Leiter EH, Cyopick P, Danska JS, Delovitch TL. Interleukin 4 reverses T cell proliferative unresponsiveness and prevents the onset of diabetes in nonobese diabetic mice. J Exp Med 1993;178:87-99.
45. Pennline KJ, Roque-Gaffney E, Monahan M. Recombinant human IL-10 prevents the onset of diabetes in the nonobese diabetic mouse. Clin Immunol Immunpathol 1994;71:169-175.

Chapter 4

EVIDENCE FOR A DEFECTIVE MATURATION AND FUNCTION OF ANTIGEN PRESENTING CELLS IN TYPE 1 DIABETICS. A FACTOR OF IMPORTANCE IN THE DISTURBED TOLERANCE TOWARDS β CELL ANTIGENS?

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SUMMARY

Type 1 diabetes is an autoimmune disease in which β cells are destroyed by autoreactive T cells and macrophages. The disease is based on a dysbalance in immunoregulation leading to an array of humoral and cellular autoimmune responses to islet cell antigens. There are numerous indications that dendritic cells - the antigen-presenting accessory cells (APCs) par excellence - play an important role in the initiation of the islet cell directed autoimmune response.

In type 1 diabetic patients we established:

1. a lowered maturation of DCs from their precursors in the blood, and
2. a lowered function of blood-derived dendritic cells (DCs), viz. a lowered capability of the cells to act as stimulator cells in autologous and allogeneic T cell stimulation and a lower capability to form cellular clusters with other lymphoid cells.

These defects were independent from the duration of the disease. Similar and innate abnormalities in the function of APC have been demonstrated in the animal models of type 1 diabetes (NOD mouse, BB rat).

Since DCs are of crucial importance in the triggering of naive T cells, and since optimal APC function is earlier required for tolerance induction than for immunization, we hypothesize that the found suboptimal maturation and function of DCs form the basis for a disturbed regulation of T cell activation in which induction of tolerance is stronger affected than induction of immunization.

Type 1 diabetes is an autoimmune disease in which islets of Langerhans are infiltrated by lymphoid cells that destroy β cells. The disease is based on a dysbalance in immunoregulation and accompanied by an array of cellular and humoral immune responses to a variety of islet cell autoantigens.

The pathogenesis of autoimmune insulin-dependent diabetes is multifactorial, and involves interactions between MHC-linked and non-MHC (HLA-DR3/DR4)-linked genes[1,2]. However, these polygenic interactions are not sufficient to precipitate active disease. The pathogenesis is also strongly influenced by environmental factors such as viruses and toxins. Effector T cells of both CD4 and CD8 phenotype, that are specific for islet cell antigens, as well as macrophages are the major cell types involved in β cell destruction: in animal models of the disease – the NOD mouse and the BB rat – $CD4^+$ and $CD8^+$ T cell transfers are able to accelerate the disease, while elimination of macrophages greatly reduces the incidence of insulitis[1,2,3]

The long subclinical period and the "honeymoon period" of type 1 diabetes suggest that the dysbalance in immunoregulation and the aggressive autoimmune response directed to the β cell can be modulated. Indeed, blood leucocyte analysis from diabetic patients suggests that there are increased numbers of regulatory T cells with a suppressor-inducer phenotype before diabetes onset, while the numbers of such cells diminish at the time of outbreak of the disease[2]. In the NOD mouse, "diabetes-protective" regulator T cells have also been found[4]. These cells are $CD4^+$, and their numbers can be augmented by various experimental regimens that reduce the incidence of diabetes, such as IL-1-, IL-2- and TNF-α-treatment and the administration of complete Freund's adjuvants[5].

The generation of regulatory and effector T cells is induced by stimulation with APCs. Amongst the APCs the dendritic cell (DC) is unique, in that it is the only APC capable of stimulating naive T cells[5,6,7]. DCs play an important role in the initiation of autoimmune responses: they are the first cells to arrive in thyroid glands or around islets, previous to any T cell or scavenger macrophage stimulation or infiltration[8]. Furthermore, DCs are able to transfer experimental autoimmune diseases such as thyroiditis and encephalomyelitis with great efficiency[7].

Conversely, DCs also have the potential to control the autoimmune response by a variety of mechanisms[5,7]. DCs are the stimulators of syngeneic mixed leucocyte responses (SMLR). The SMLR, which is depressed in NOD mice, is considered to be important in the generation of regulatory suppressor T cells[2,4]. Recently, it has been demonstrated that injection of pancreatic-lymph-node DCs in the foot pads of 4 week old NOD mice is able to prevent the development of diabetes, and generates transferable diabetes-protective T cells[5].

The apparent discrepancy between DCs as stimulators or downregulators of immune responses has been explained by antigenic load: DCs pulsed with a high concentration of tumour antigen inhibit immune responses to that antigen, whereas pulsing with low antigen concentrations stimulates the immune response[7]. Apart from playing a role in the generation of regulatory T cells, DCs also participate in the selection of the T cell repertoire in the thymus. Collectively, these data show that DCs have the potential to regulate endocrine autoimmune responses.

Characteristic for DCs are the active movements of the cell and their capability of seeking contact with any other cell in their vicinity. In doing so DCs form cellular aggregates, which are relatively short-lived[9]. This aggregate formation is temperature-dependent (37°C) and various adhesion-molecules are operative. When this early clusterformation occurs between

a DC and a T cell, and when such a T cell recognizes an antigen in the context of the MHC complex on the membrane of the DC a stable cluster between the DC and the T cell is formed[9]. Such a stable clusterformation between DCs and T cells lasts for several days, is temperature-independent (4°C) and results in the production of lymphokines. The development of responsiveness of lymphocytes to these lymphokines and the initiation of a mixed leucocyte reaction (MLR) occurs in such stable clusters. Consequently, it has been found that the numbers of DCs and formed stable clusters in MLRs correlate well with T lymphocyte stimulation[6,9].

To look for a possible disturbance in the function of DCs in human type 1 diabetes, we have studied the maturation of DCs from precursor cells in suspensions of peripheral blood monocytes and the potential of blood DCs:
a) to act as stimulator cells in autologous and allogeneic MLR's and,
b) to form temperature-dependent early cellular aggregates.

Type 1 and type 2 diabetic patients (disease controls) were recruited from the Pediatric and Internal Medicine Department of the Free University Hospital, Amsterdam, the Internal Medicine Department of the Spaarne Hospital, Haarlem and the Pediatric (Sophia Children's Hospital) and Internal Medicine Department (University Hospital Dijkzigt) of the Erasmus University, Rotterdam. Patients with complications of their diabetes (such as infections), or with other concomitant diseases, or patients undergoing other medical treatments in addition to that for diabetes were excluded. The type 1 diabetic patients of whom blood was drawn for testing (n = 61, ages 3-58 year, ♂:♀ = 1.7:1.0) were all treated with insulin, apart from 2 recently discovered diabetics of whom blood was drawn before insulin treatment. The type 2 diabetic patients (7 males, 6 females, ages 45-70 years) had long-standing diabetes, and were on insulin treatment following a long period of oral antidiabetic therapy.

Healthy young laboratory staff (16 females, 15 males, ages 24-31 years) and older blood bank donors (6 females, 6 males, ages 50-65 years) served as healthy controls. Tests for presence of islet cell antibodies (ICA) in the serum of the patients were performed routinely using the standardized indirect immunofluorescence methods for ICA detection.

DCs were obtained from the peripheral blood of the diabetes and controls via two procedures. In the first procedure DCs were prepared from blood mononuclear cells (Ficoll-Isopaque densitity gradient centrifugation) via adherence steps to plastic according to the method of Knight et al[7].
In the second procedure DCs were maturated from monocyte suspensions (Percoll gradient

centrifugation) via pulsing with the thyroid hormone triiodothyronine (1h, 37°C) followed by overnight non-adherent culture. This procedure has recently been described in detail[6], and yields relatively high numbers of DCs. It has been verified that DCs obtained via this latter procedure have the same marker pattern as DCs isolated via the "Knight procedure" and are as excellent stimulatorcells in MLR[6]. Numbers of triiodothyronine (T3) induced monocyte-derived DC were counted in wet preparations (cells with a dendritic morphology), and cytospin preparations (DCs positive for MHC-class II markers and negative for acid phosphatax)[6]. In fourteen patients and controls the monocyte-derived DC were also tested for their stimulator capacity in autologous and allogeneic MLR via earlier described standard procedures (in short: ratio irridiated DC : T cell = 1:5, total volume 200µl RPMI 1640, 10% blood group A serum, antibiotics, 5 day culture)[6]. As responder cells T cells were used that had been isolated via standard procedures using nylon wool adherence[6].

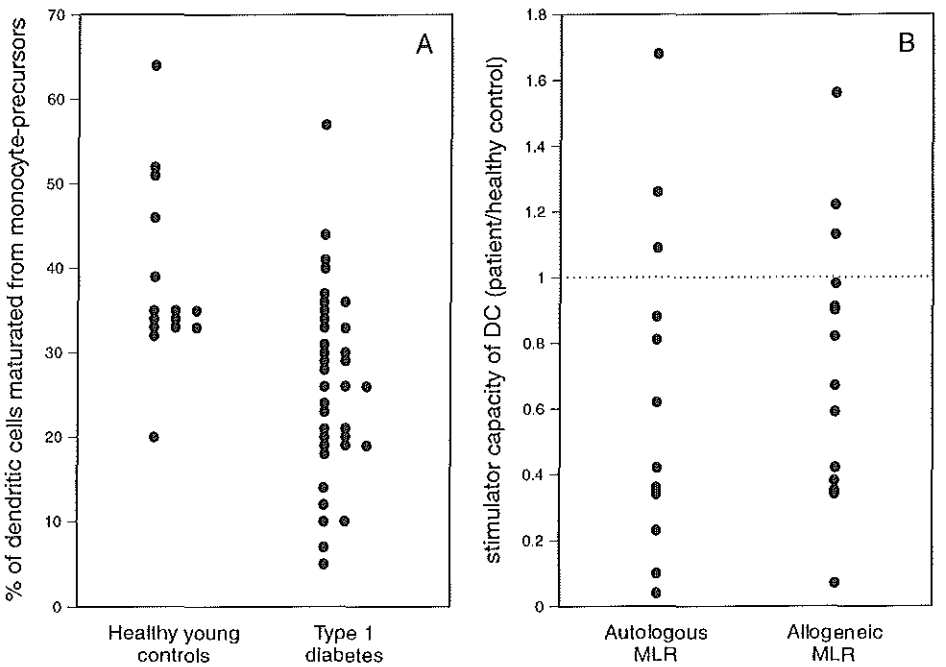

Figure 1a. The % of dendritic cells (DC) maturated from blood monocytes after exposure to T3 (1 hr, 37°C) and overnight culture under nonadherent conditions (37°C, 5% CO_2). Note that fewer DCs were found when type 1 diabetic monocytes were used ($p = 0.0005$, Mann Whitney test).

Figure 1b. The stimulator capacity of DC (as obtained in a. via the exposure to T3 and overnight culture of monocytes) was assessed. Irridiated DC were used as stimulatorcells in autologous and allogeneic MLR with purified T cells as responder cells. Stimulation was assessed by ^{3}H-thymidine incorporation into T cells. Since there were considerable inter-assay-variations, data obtained with diabetic DCs were expressed in relation to data obtained with healthy control DCs in the same assay (the latter is 1.0). Note that the stimulator capacity of diabetic DC is lower both in autologous and allogeneic MLR (viz lower than 1.0;$p < 0.05$, Mann Whitney paired two tailed test).

In type I diabetics numbers of DC that could be induced from precursors in the blood monocytes suspensions were lower: viz 26.2% (± 11.0 standard deviation, n = 36) in type 1 diabetics versus (vs) 38.4% (±10.3%, n = 15) in the young healthy controls (fig. 1a, difference statistically significant, p = 0.0005, Mann-Whitney two tailed test).
Not only was the induction of DC from their precursors defective in type I diabetics, the DCs that had been induced, were less capable to act as stimulator cells for both autologous and allogeneic T-lymphocytes, and values of ^{3}H-thymidine incorporation into proliferating T cells were 65.2% (autologous, mean, sd 48.2%, n = 12) and 73.8% (allogeneic, mean, sd 39.6%, n = 14) of the control value (the ^{3}H-thymidine incorporation into the T cells when stimulated with DC of a healthy subject, see figure 1b).

The defective stimulator function of the DC of type 1 diabetics was also reflected in a diminished capability to form temperature-dependent non-antigen-specific cellular aggregates. In the assays for cellular aggregate formation blood-derived DCs were used that were either isolated via the Knight procedure or via the nonadherent culture of bloodmonocytes after T3-pulsing. DCs obtained in the "Knight procedure" ($5x10^4$) were allowed to cluster with $5x10^3$ autologous T cells, whereas DCs obtained in the "T3-pulsed" procedure ($5x10^4$) were allowed to cluster with $5x10^4$ autologous T cells (an excess of DCs in comparison to other lymphoid cells gives larger and more easily detectable cellular aggregates). After incubation (4h, 37°C, 5% CO_2) in 250 µl flat-bottomed wells the formed cellular clusters were counted using an inverted microscope and numbers were expressed as the number of clusters per six microscopic fields (magnification x200). A cluster was defined as an accumulation of 4-25 cells. Cytospin preparations of the cell clusters after 4 h incubation revealed that the cell aggregates both in patients and healthy controls consisted of cells with a dendritic morphology and lymphocytes, but dendritic cells were always in the majority. Cluster formation did not take place in the absence of dendritic cells or when incubated at 4°C. The cells with dendritic morphology involved in the aggregates could be identified as classical DC: they were strongly positive for class II MHC determinants, had a reniform nucleus and little or no acid phosphatase activity.

The number of *in vitro* formed clusters was considerably smaller in type 1 diabetics as compared to healthy controls irrespective of the method used to isolate the DCs (see table). The DCs of type 2 diabetic patients had a cluster capability comparable to that of healthy controls. Since all of the type 2 diabetics were on insulin treatment, we deduce that iatrogenic insulin administration has no effect on DC cluster capability. The two patients with recent onset diabetes without insulin treatment also had a defective cluster capability. We did not find a relationship with Hb1AC levels.

It is also worthy to note that the diminished maturation of DCs from blood precursors and their diminished function (stimulation in MLR and non-antigen dependent cluster formation) was found irrespective of the duration of the disease (see table). There was also no relation to age or sex of the patients. Of ICA-positive patients, 69% had a defective cluster capability (viz. < 100 clusters per 6 microscopic field x200); of ICA negative patients, 50% demonstrated defects in cluster capability (difference not statistically significant).

Table 1

ISOLATION PROCEDURE	CELLULAR AGGREGATES per 6 microscopic fields (x 200 magn.)		
	Study group	Number	Mean (s.d.)
Dendritic cells induced from monocytes after T3 pulse and overnight culture.	Healthy controls (young)	11	135 (41)*
	Type 1 diabetics	27	90 (45)*
"Knight-procedure"	Healthy controls (young)	16	193 (64)*
	Healthy controls (old)	11	176 (60)*
	Type 2 diabetics	13	154 (84)*
	Type 1 diabetics		
	- all	25	103 (76)*
	< 2 yrs duration	6	116 (84)
	2-10 yrs duration	12	97 (68)
	> 10 yrs duration	7	104 (80)

The non-antigen dependent cluster capability of blood-derived dendritic cells (DC). DC were of type 1 diabetics, type 2 diabetics and healthy controls (young = 24-31 yrs; old = 50-65 yrs). For technical details of the experiment see ref 6. Note the lower ability of type 1 diabetic DCs to form cellular aggregates irrespective of the method of isolation or the duration of the disease.
(* = statistically significant, $p<0.01$, Mann Whitney two-tailed-test)

Our data of a lowered stimulator capability of blood DC of type 1 diabetics for both autologous and allogeneic T cells partly corroborate earlier findings by Faustman *et al.*[2]. They found a defective autologous mixed leucocyte culture (AMLRs) in type 1 diabetics. These defective AMLRs (reflecting the defective stimulation of autologous regulatory T cells[2,4]) were in the hands of these authors dependent on the presence of both diabetic APCs and diabetic T cells. Allogeneic MLRs, in which diabetic APCs or T cells were either cocultured with healthy T cells or healthy APCs, gave normal proliferative responses. In our hands also allogeneic MLRs (patient APC and healthy T cells) were defective.

The defective maturation and function of DCs was detectable in type 1 diabetics more than 10 years after the onset of the disease, and in the same proportion as in recent type 1 diabetics (see table). This strongly suggests that the defect in APC-function is not related to insulitis activity. There are indications that there exist in autoimmune diabetes maturational abnormalities in the macrophage- and APC lineages at the level of the bone marrow[10]: In the NOD mouse, progenitor cells of granulocytes/monocytes are deficient in their *in vitro* responses to IL-3, GM-CSF, IL-5 and CSF-1[1,10]. Due to these defects, less mature cells are generated. Not only are there developmental defects in NOD mice; peritoneally-derived APCs of NODs also have a decreased ability to activate cytotoxic T cells, due to an aberrant MHC class I induction under the influence of γ-IFN[2]. NOD peritoneal macrophages also show an aberrant arachidonic acid metabolism[3]. In type 1 diabetic patients, a generalized defective infiltration of DCs into the skin has been described[8]. Collectively, these data indicate that there are defects in the myeloid differentiation of autoimmune diabetic individuals that generate fewer and functionally aberrant APCs. In NODs, these maturational defects are partly determined by the diabetogenic H2g7 haplotype and partly by other diabetogenic genes.

How is this defective maturation and function of APCs related to an enhanced β cell autosensitization? It has been described that antigens that are processed and presented in an inefficient fashion can continue to stimulate T cell proliferative responses in the periphery, but are unable to induce tolerance[5]. Similarly, it has been demonstrated that activation-driven T cell death requires quantitatively more antigenic stimulation than is required to trigger T cell proliferation[5]. In addition, the stimulation of immunoregulatory T cells requires a more highly activated APC than is required to activate effector T cells[5]. Collectively, these findings indicate that the threshold of T cell activation required to reach the state of tolerance is much higher than that necessary to trigger an effector response in the periphery. We therefore consider the defects, as found by us in the maturation and function of diabetic DCs, as abnormalities in APC function that affect tolerization earlier than (auto)immunization.

The above-described view on the role of DCs in type 1 diabetes opens new avenues for the treatment of the disease, since vaccination with certain populations of DCs has been proven to be useful to actively tolerize prediabetic NOD mice[4].

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REFERENCES

1. Serreze DV, Gaskins HR, Leiter EH. Defects in the differentiation and function of antigen presenting cells in NOD/Lt mice. J Immunol 150:2534-2543 (1993).
2. Faustman D, Li X, Lin HY, Fu Y, Eisenbarth G, Avruch J, Guo J. Linkage of faulty major histocompatibility complex class I to autoimmune diabetes. Science 254:1756-1761 (1991).
3. Lety M-A, Coulaud J, Bens M, Dardenne M, Homo-Delarche F. Enhanced metabolism of arachidonic acid by macrophages from non-obese diabetic (NOD) mice. Clin Immunol Immunopathol 64:188-196 (1992).
4. Serreze DV, Leiter EH. Defective activation of T suppressor cell function in non-obese diabetic mice. J Immunol 140:3801-3807 (1988).
5. Clare-Salzler MJ, Brooks J, Chai A, Van Herle K, Anderson C. Prevention of diabetes in non-obese diabetic mice by dendritic cell transfer. J Clin Invest 90:741-748 (1992).
6. Mooij P, Simons PJ, de Haan-Meulman M, de Wit HJ, Drexhage HA. The effect of thyroid hormones and other iodinated compounds on the transition of monocytes into veiled/dendritic cells. A role of GM-CSF, TNF-α and IL-6. J Endocrinol 140:503-512 (1993).
7. Knight SC, Hunt R, Dorse C, Medewar PB. Influence of dendritic cells on tumour growth. Proc Natl Acad Sci USA 82:4495-4497 (1985).
8. Voorbij HAM, Jeucken PHM, Kabel PJ, de Haan M, Drexhage HA. Dendritic cells and scavenger macrophages in pancreatic islets of prediabetic BB rats. Diabetes 38:1623-1629 (1989).
9. Inaba K, Steinman RM. Accessory cell T lymphocyte interactions. Antigen-dependent and antigen-independent clustering. J Exp Med 163:247-261 (1986).
10. Langmuir PB, Bridgett MM, Bothwell ALM, Crispe IN. Bone marrow abnormalities in the non-obese diabetic mouse. Int Immunol 1993;5:169-177.

Chapter 5

GENERAL CONCLUSIONS AND DISCUSSION

5.1 Conclusions that can be drawn from the described experiments

The immunohistochemical studies described in this thesis confirm that macrophages and dendritic cells (DC) are involved in the development of type I diabetes. The studies clearly show that there are various subclasses of macrophages and DC playing a role in the initiation of the insulitis process and in the destruction phase of the β-cells. More specifically: before lymphocyte infiltration is detectable around the islets, an accumulation of ER-MP23$^+$, MHC class II$^+$ dendritic-shaped cells and of MOMA1$^+$ macrophages, both of which are assumed to exert an APC function, can be demonstrated near the islets of NOD mice (chapter 3.1). Very recent experiments in our lab using the monoclonal antibody N418, reacting with CD11c$^+$, which is mainly present on DC, show that this antibody is particularly suited to stain DC *in* NOD islets: N418$^+$ DC inside the islets clearly augment in number in the initial stages of the insulitis process before lymphocyte accumulation. Also at the circumference of the islet, the number of N418$^+$ DC rises in the initial stages of the insulitis. The N418$^+$ DC at the circumference of the islet are probably partly overlapping with the ER-MP23$^+$ DC described in the studies in this thesis.

The NOD islets are larger than those of BALB/c mice (chapter 3.2). NOD-*scid/scid* mice, which do not develop diabetes, since they lack functional B and T cells, also show such larger pancreatic islets and an accumulation of antigen presenting cells (APC) around these islets (chapter 3.2). Prophylactic treatment with exogenous insulin during these early phases reduces the abnormally large islet sizes in the NOD as well as the number of the accumulating macrophages and APC and results in a lower incidence of diabetes. It is therefore likely that the early accumulation of APC is related to the metabolic activity of islet cells (chapter 3.2).

In the later phases of the NOD insulitis process, when lymphocyte accumulation around the islets has been established, infiltration by cytotoxic/phagocytotic BM8$^+$ macrophages into the islets can be demonstrated, which is the more prominent in situations with a more severe and rapid loss of β-cells, namely female NOD mice and castrated male NOD mice (chapter 3.4). Since NOD-*scid/scid* mice do not show an insular infiltration by this type of macrophages (chapter 3.2), infiltration by these macrophages is probably related to effects exerted by the specific B cell and/or T cell system. What underlies this infiltration can not be revealed from our data, but there are a few possibilities. Firstly, T cells may provide an activation signal, inducing these macrophages to destroy β-cells. Such an activation signal might be IFN-γ, produced by Th1 type cells[1]. Secondly, antibodies might coat the β-cells, activate complement, thereby attracting and activating macrophages. Thirdly, the T-cells and B-cells accumulated around the islets might themselves generate β-cell toxic products (IFN-γ again is a good candidate[2]), resulting in β-cell death, which would require macrophage

infiltration to clear the debris. It is also feasible that combinations of these mechanisms play a role.
Our data further show that the milieu of the para/peri-insulitis is important for the progression to diabetes and β-cell loss. Chapter 3.3 suggests that protective T cells are generated after intrathymic islet cell transplantation, that accumulate around the islets but change the cytokine milieu in such a way that $BM8^+$ macrophages do not infiltrate the islets. On the other hand, castration (androgen-deprivation) of NOD males probably may change the cytokine milieu in an opposite way, resulting in a faster progression of infiltrative insulitis (chapter 3.4). A case of human type I diabetes with a particular fulminant progression was histologically similar to the insulitis seen in castrated NOD males (chapter 2).

The studies performed on human monocyte-derived dendritic cells (chapter 4) demonstrate that DC from type I diabetic patients show various functional abnormalities. Maturational defects, defects in the formation of clusters with neighboring cells, and a reduced capacity for T cell activation all indicate a reduced effectiveness in the presentation of autoantigens to the immune system. Such an ineffective auto-antigen presentation is hypothesized as underlying the loss of tolerance[3]. It has been demonstrated that the threshold of T cell activation required to induce tolerance is much higher than that required to trigger an effector response in the periphery[4]. Moreover, stimulation of immunoregulatory T cells requires a more highly activated APC[5] and a more antigen-loaden APC[6] than is required for triggering of effector T cells. An interesting observation in this respect is that a decreased activity of suppressor cells is commonly found in IDDM patients[7,8]. Also in the NOD mouse it was found that the syngeneic MLR (SMLR), which is described to generate cells with a suppressor function[9], was depressed and that the cells generated in this SMLR did not possess their normal ability of suppressing an allogeneic MLR[10]. A defective production of IL-1β in the NOD probably plays a role in this disturbance of the SMLR, since supplementation of this cytokine in the SMLR partly restored the defective reaction[10]. Disturbed maturation of NOD-macrophages may explain the defective production of IL-1β in he NOD SMLR, since macrophages do not acquire the ability to produce IL-1β until the later stages of differentiation[11]. Serreze et al demonstrated that NOD mice indeed show an abnormal macrophage development and maturation[12,13] and that this defect is associated with a genetically determined decreased ability of NOD macrophages to up-regulate CSF-1 receptor and IFN-γ receptor upon incubation with CSF-1[13]. Moreover, after up regulation of these receptors with the help of IFN-γ these receptors were found to have a greatly reduced ability to activate protein kinase C-coupled second messenger pathways[13].
Another functional disturbance in NOD macrophages, an intrinsic higher production of

$PGE_{2\alpha}$[14], was recently shown to down regulate the cluster-capability of DC in co-culture[15]. In line with these experiments it has been described that *macrophage-free* DC from the pancreas draining lymph nodes of the NOD mouse do cluster effectively with T cells from the same lymph nodes[16]. When these *macrophage free* DC from pancreas draining lymph nodes, or *macrophage free* NOD DC incubated with a high concentration of islet cell antigens are injected into the footpad of 4-week-old female NOD mice, these DC, loaden with high amounts of β-cell antigen, are able to protect NOD mice from developing diabetes[17]. In addition, this protection is transferable with lymph node cells from these mice[17]. So the probably high concentration of antigen on the injected NOD DC together with an absence of NOD macrophages may overcome their impaired capacity of inducing diabetes protective T cells.

In conclusion, there firstly is an elevated number of macrophages and DC accumulated around the islets in early stages of insulitis development, which is probably due to factors produced by metabolically disturbed β-cells. Secondly, the function of the APC present around the islets is disturbed, which might be due to a negative influence of the also accumulated macrophages. The presence of functionally impaired APC results in a preferential generation of effector T-cells, at the expense of regulatory T-cells with a diabetes protective ability.

5.2 A hypothetical model for the pathogenesis of type I diabetes

Of almost every factor potentially involved in a β-cell directed autoimmune reaction (schematically indicated in figure 1), defects or abnormalities have been described, either in the human disease, in the NOD mouse and/or in the BB rat, that might contribute to the progression of the autoimmune reaction and destruction.

Islet vessel:

Before lymphocytes infiltrate around the islets, NODs show abnormal vessels close to the islets, with a high endothelium and an enlarged peri-vascular area (chapter 3.1). Also in BB rats defects of the pancreatic vessels have been described, resulting in an abnormal leakiness of the vessels[18]. It is easily conceivable that such abnormalities may lead to an enhanced infiltration of macrophages, DC, and other inflammatory cells.

β-cell:

Islets in the NOD mouse are large. They are probably metabolically disturbed and attract macrophages and DC in abnormal numbers (chapter 3.2). Similar abnormalities exist in the NOD-*scid/scid* mouse. Also in the human a β-cell abnormality may be present since first degree relatives of type I diabetic patients have been described to have an elevated fasting pro-insulin level[19,20]. The same abnormalities in β-cell activity may also lead to a higher vulnerability of the β-cell for β-cell toxic products[21] (like NO and IL-1).

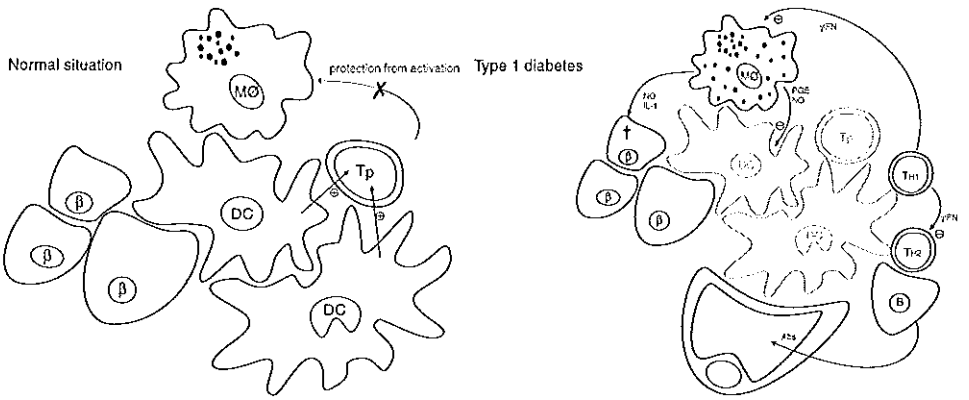

Figure 1. Schematic representation of events taking place in the course of the β-cell directed autoimmune response. A high endothelium of the vessels near the pancreatic islets, in combination with an abnormal β-cell metabolism results in the accumulation of high amounts of functionally disturbed APC. This functional disturbance results in a preferential generation of effector T cells, at the expense of regulatory T cells with a diabetes protective ability. A maturational and functional disturbance of macrophages may contribute to the functional impairment of the APC. Finally, macrophages activated by effector T cells produce β-cell toxic factors, for which these metabolically disturbed β-cells are extremely sensitive.

Dendritic cells and macrophages:

Maturational and functional disturbances are found in DC from human type I diabetic patients (chapter 4, ref[22]), as well as in APC of the NOD mouse[10,12]. In the NOD mouse these maturational and functional abnormalities are important in the development of the disease, since diabetes does not occur in animals corrected for these disturbances in their APC function[23]. Maturational disturbances have also been described for NOD macrophage lineages[13]. Functionally, NOD macrophages show a high basal level of production of prostaglandins and leukotriens as compared to normal strains[14]. The high production of these factors may result in down regulation of the APC function of DC.

Lymphocytes:

There are reports of lower numbers of regulatory T cells as compared to effector T cells in the NOD mouse[24] as well as in the human[7,8]. Since regulatory T-cell stimulation can be restored in the NOD by regimens which positively influence the function of APC[25], these findings are probably secondary to APC dysfunctions as described above.

The finding of these different disturbances on various levels in the β-cell directed autoimmune response is compatible with the recently gathered insights that IDDM is polygenic[26] and multifactorial. It is also not surprising that various environmental factors, like the influence of temperature, diet, viruses and chemical compounds disturb or modulate the autoimmune response on these various levels. In fact, two major dysregulations can now be identified that may be regarded as tumblers in the key allowing IDDM to develop (figure 2). One is immunodysregulation (a systemic factor), the other is a pre-existent β-cell or endothelial cell abnormality (a local factor).

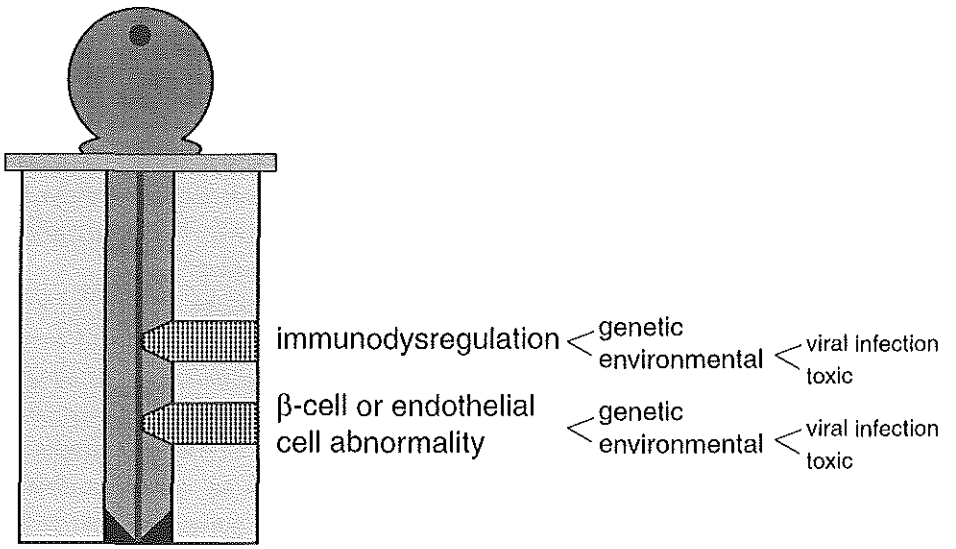

Figure 2. The key for the development of diabetes. Two tumblers need to be moved. The first tumbler is immunodysregulation, a systemic factor. The second tumbler is a local factor, leading to an enhanced infiltration of the pancreas by these disturbed immune cells. Both tumblers can be affected by genetic, infectious, and toxic influences.

The first tumbler: immunodysregulation

Immunodysregulation may result from genetic abnormalities, as described for the NOD mouse. Also, at least in part of the human cases of diabetes type I, genetic components play a role, since IDDM tends to be a familiar disease in many cases.

Not only genetic components may be responsible for disturbances in the regulation of

the immune system. Also viral infections, and especially retroviruses, may modify immune cell function. In some cases this modification leads to the expression of features of autoimmunity[27]. Many infectious retroviruses cause immunosuppression or have other immune effects. At least some of these effects are attributable to direct effects of retroviral proteins. For example, the HIV envelope protein, gp120 stimulates the production of IL-1 and arachidonic acid by human monocytes[28]. Moreover, the synthetic peptide CKS-17, which contains a highly conserved amino acid region of the transmembrane retroviral envelope protein p15E has been described to inhibit protein kinase C (PKC) activity, which has been implicated in mediating IL-1 responses[29]. Note that also the NOD mouse was recently described to have a genetic defect in PKC activity, that resulted in maturational defects of its APC[12].

Though human IDDM has been associated with congenital rubella virus infection (33% of children with congenital rubella are diabetic[30]) and postnatal infection by Cocksackie B4-virus[31] it is still not known whether immunoregulatory defects induced by these viruses play a role. The finding of elevated numbers of both $CD4^+$ and $CD25^+$ (IL-2 receptor) T cells with an abnormally low release of soluble IL-2R in children with congenital rubella syndrome[32] suggests that immunodysregulation may be induced by these viruses.

Apart from immunodysregulation caused by genetically programmed defects in immune cell function or caused by viral infection, toxins may also play a role. Cyclophosphamide (CY) was described to have a deteriorating effect on suppressor mechanisms in NOD mice resulting in a rapid β-cell destruction in mice transplanted with fetal pancreas isografts after administration of CY 3 days before the transplantation[33].

Streptozocin, which is known to induce IDDM in certain mouse strains[34], is also described to have effects on the immune system. In general, depression of immune reactivity has been reported, including a reduced proliferative response to Concanavalin A. It has been described that the suppressor cell population activated in the autologous MLR is equivalent to that activated in response to Con A[35]. Hence, streptozocin might have an effect on the ability of the immune system to induce suppressive responses. Moreover, streptozocin was demonstrated to induce a higher activation state of the macrophages[36]. In addition to the higher production of β-cell toxic products by such activated macrophages, a high activation state of these macrophages may also have negative effects on DC function.

The second tumbler: a β-cell or endothelial cell abnormality

A β-cell or endothelial cell abnormality may lead to enhanced DC and macrophage infiltration starting off the immune reaction. β-cell abnormalities may additionally lead to an enhanced β-cell vulnerability to the autoimmune attack.

The β-cell abnormalities and/or endothelial cell abnormalities may again be based on

genetic components and/or environmental factors. In the NOD as well as in the NOD-*scid/scid* mouse an abnormal histology of both the islet vessels and the islets was demonstrated in combination with an elevated number of infiltrating APC (chapter 3.2).
In addition, infections may also alter β-cell metabolism. Viral infections of β cells are generally regarded to be able to alter β-cells, thereby either affecting insulin production, causing abnormal antigenic determinants to appear on the β-cell surface[37,38], or leading to an increased production of proinflammatory cytokines or chemoattractants[39]. Elevation of the adhesion molecules on the vessel endothelium and leakiness of the vessels are well established steps in the inflammation process.
Again, also toxins may alter the β-cells in a way that they become recognizable by the immune system, and thus may attract larger numbers of APC. Streptozocin in low doses has been described to trigger an auto-immune response towards the β-cells. Sequential studies revealed that the first cells attracted to the islets belong to the macrophage lineages[40,41]. This infiltration appeared to be essential for the later development of diabetes, since blockade of macrophage influx by silica was described to abolish also the lymphocytic component of the insulitis and the development of diabetes[42]. These studies also showed that islets release a macrophage-chemotactic lipid after low-dose streptozocin[41].
In preliminary studies in the NOD-*scid/scid* mouse treated with very low doses of streptozocin once a day from day 1 to 5, we found that an initially rapid but limited β-cell loss coincided with a fast infiltration into the islets by $BM8^+$ macrophages, somewhat later followed by infiltration with Mac-1^+ macrophages. After the end of the treatment at day 5, the β-cell loss stopped temporarily, and progression of further β-cell loss was found only after massive infiltration of macrophages into the islets (see figure 3). The early, limited, β-cell loss is suggestive of a minor initial β-cell damage or alteration which gives rise to attraction of macrophages into the islets. The later phase of β-cell loss, after the massive infiltration of macrophages, may be explained by several mechanisms. For example, β-cells damaged or altered by streptozocin may be more vulnerable to macrophage products, macrophages may be activated by streptozocin and produce higher amounts of β-cell toxic products. Also a combination of these mechanisms might lead to the second phase of β-cell loss.
As has been suggested above, β-cell factors that initiated the higher attraction of DC and macrophages may also render them more vulnerable to the autoimmune attack: if β-cells are genetically predisposed to an elevated metabolism, which may attract APC, the higher activity state of the β-cells will also make them more vulnerable to β-cell toxic products like IL-1β[43].

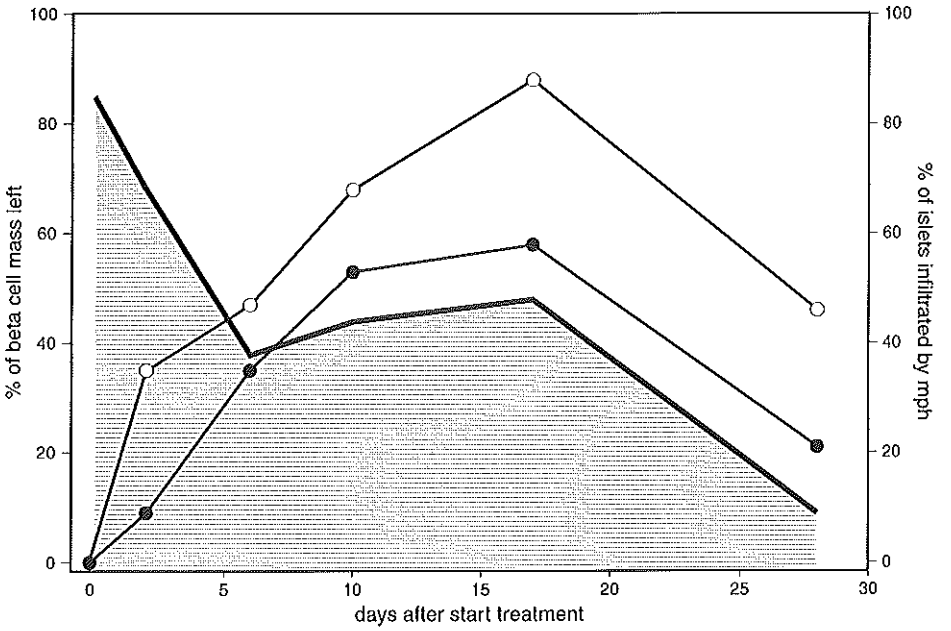

Figure 3. Effect of very-low dose streptozocin treatment in 7 weeks old male NOD-*scid/scid* mice on macrophage (mph) infiltration and β-cell loss. Mice were injected with 30 mg/kg streptozocin on five consecutive days (day 1-5). Infiltration by $BM8^+$ macrophages (○), and $Mac\text{-}1^+$ macrophages (●), as well as the percentage of $insulin^+$ cells per islet mass were assessed on days 0, 2, 6, 10, 17, and 28 after the start of the treatment.

Virus-infection may also induce β-cells to become more vulnerable[37]. Cocksackie B4 virus has been reported to have direct cytotoxic effects on the β-cell[44].

Finally, **toxins** can elevate β-cell vulnerability. A well known compound doing so is streptozocin, again. Streptozocin causes DNA strand breaks, and at the same time alkylates cell components necessary for the generation of ATP. DNA repair enzymes, which use large amounts of ATP, will start to repair the DNA strand breaks. Since ATP generation is blocked by alkylation of mitochondrial enzymes, this results in a fast depletion of cell energy resources, which leads to cell death.

Thus, the two major tumblers can be touched by different factors. Different combinations of factors, provided they result in a fitting key, will all allow the key to be turned and diabetes to develop.

It is special that a few factors or components are able to influence both tumblers, as has been described for streptozocin and Cocksackie B4 virus. Streptozocin and Cocksackie B4 influence the immune system, the attractability of the β-cell for APC and the vulnerability of the β-cell for immunocytotoxic components. This might explain the

supreme diabetogenic ability of these compounds. It goes without saying that also a combination of genetics (for instance immunodysregulation), a viral infection (an insulinotropic virus expressed on the β-cell), and a chemical substance in the diet later (that renders the β-cell vulnerable to immune attack) may result in diabetes.

5.3 Consequences of this hypothetical model for the pathogenesis of type I diabetes for therapeutic programs

Correction of the first tumbler: immunoregulation

This option has recently been extensively investigated, particularly in animal models and a few approaches seem to be successful.

1. In NOD mice transplantation of syngeneic islets into the thymus is a therapy with a high success rate[45]. Before it can be used in human patients, problems have to be solved. Syngeneic islets cannot be used in humans, so it should be investigated if allogeneic islets, which may be obtained from cadaveric donors, give the same result. Besides, introduction of virus infection is a risk which has to be considered, and also logistic problems in obtaining the islets can be expected. Therefore, experiments with (combinations of) peptides from islet components are done to circumvent these problems. Also another problem may need to be solved in adult patients: the thymus in adults may sometimes be reduced to not much more than a small streak of fibrous tissue. The effectiveness of such a rudimentary thymus in the prevention of diabetes can be doubted. The largest part of the patients needing prevention, however, will be children and adolescents, in which the thymus is still present.
2. BCG-vaccines to up regulate immune functions have been investigated both in NOD mice and in human patients. In the human situation it is described to prolong the honeymoon period of recently diagnosed patients[46]. Results of this therapy in pre-diabetic patients have not yet been described.
3. Another way to up regulate immune functions would be administration of cytokines. Indeed, activation of the immune system using various cytokines like IL-1, IL-2, TNF-α and/or IFN-γ has been described to effectively prevent diabetes in the NOD mouse[23,47], but because of the many side-effects this is not an option in human diabetes at the moment.
4. Vaccination with *macrophage-free* DC from the pancreas draining lymph node has been successful in preventing diabetes in NOD mice[17]. At the moment it is investigated if DC from other sources, pulsed with β-cell antigens give the same results.
5. Finally, tolerance induction via injection of GAD-peptides[48], or the β-chain of insulin[49] have recently been shown to prevent diabetes. This is highly

experimental at the moment, since the requirements for a tolerance induction as opposed to an effector response have not yet been fully defined in the human situation.

Correction of the second tumbler: downregulation of β-cell metabolism

Treatment with exogenous insulin downregulates abnormal β-cell metabolism, thereby reducing the number of APC attracted to the islets, and also diminishing the vulnerability of β-cells to toxic products. Small trials have already shown a significant reduction in diabetes incidence in the first 2 years of treatment[50]. There is, however, a problem to be expected in applying this therapy in children, which will be the main target group in preventive programs. It is clinical experience that it is difficult in many children to adjust the dose of insulin to their daily needs. Therefore, a relatively high dose of prophylactic insulin may bear a substantial risk for hypoglycemia, whereas a low dose might not be as effective in the prevention of diabetes. To be able to adjust the prophylactic insulin dosage more precisely, frequent screening of C-peptide levels may be necessary.

It might also be an option to combine prophylactic administration of exogenous insulin with one of the possible interventions in the regulation of the immune system mentioned before. Of these possibilities only BCG-vaccination will be available for safe application in the near future.

In conclusion, the above described hypothesis on the pathogenesis of IDDM may result in rational strategies to detect new entrances for preventive therapies.

REFERENCES

1. Campbell PA, Canono BP, Cook JL. Mouse macrophages stimulated by recombinant gamma interferon to kill tumor cells are not bactericidal for facultative intracellular bacterium *Listeria monocytogenes*. Immunology 56:1371-1375,1988
2. Rabinovitch A. Roles of cytokines in IDDM pathogenesis and islet β-cell destruction. Diabetes Rev 1:215-240,1993
3. Milich DR, Jones JE, McLachlan A, Houghton R, Thornton GB, Hughes JL. Distinction between antigenicity and tolerogenicity among HBcAg T cell determinants: influence of peptide-MHC interaction. J Immunol 143:3148-3156,1989
4. Ucker DS, Meyers J, Obermiller PS. Activation-driven T cell death. II. Quantitative differences alone distinguish stimuli triggering nontransformed T cell proliferation or death. J Immunol 149:1583-1592,1992
5. Ishikura H, Jayaraman S, Kuchroo V, Diamond B, Saito S, Dorf ME. Functional analysis of cloned macrophage hybridomas VII. Modulation of suppressor T cell-inducing activity. J Immunol 143:414-419,1989
6. Knight SC, Hunt R, Dorse C, Medawar PB. Influence of dendritic cells on tumor growth. Proc Natl Acad Sci USA 82:4495-4497,1985
7. Buschard K, Madsbad S, Rygaard J. Depressed suppressor cell activity in patients with newly diagnosed insulin dependent diabetes mellitus. Clin Exp Immunol 41:25-32,1980
8. Topliss D, How J, Lewis M, Row V, Volpe R. Evidence for cell-mediated immunity and specific suppressor T lymphocyte dysfunction in Grave's disease and diabetes mellitus. J Clin Endocrinol Metabol 57:700-705,1983

9. Yamashita U, Ono S, Nakamura H. The syngeneic mixed leucocyte reaction in mice. II. The region 1 control of suppressor T cell activity induced in the syngeneic mixed leucocyte reaction. J Immunol 128:1010-1017,1982
10. Serreze DV, Leiter EH. Defective activation of T suppressor cell function in nonobese diabetic mice. J Immunol 140:3801-3807,1988
11. Witsell AL, Schook LB. Macrophage heterogeneity occurs through a developmental mechanism. Proc Natl Acad Sci USA 88:1963-1967,1991
12. Serreze DV, Gaskins HR, Leiter EH, Defects in the differentiation and function of antigen presenting cells in NOD/Lt mice. J Immunol 150:2534-2543,1993
13. Serreze DV, Gaedeke J, Leiter EH. Hematopoietic stem-cell defects underlying abnormal macrophage development and maturation in NOD/Lt mice: Defective regulation of cytokine receptors and protein kinase C. Proc Natl Acad Sci USA 90:9625-9629,1993
14. Lety M-A, Coulaud J, Bens M, Dardenne M, Homo-Delarche F. Enhanced metabolism of arachidonic acid by macrophages from nonobese diabetic (NOD) mice. Clin Immunol Immunopathol 64:188-196,1992
15. Clare-Salzler M, Robinson P. Deleterious effects of macrophages on dendritic cell T cell interactions in the NOD mouse. Abstract presented at the 13th international immunology and diabetes workshop, 1994, Montvillargenne, France
16. Clare-Salzler, Mullen Y. Marked dendritic cell-T cell cluster formation in the pancreatic lymph node of the non-obese diabetic mouse. Immunology 76:478-484,1992
17. Clare-Salzler M, Brooks J, Chai A, van Herle K, Anderson C. Prevention of diabetes in nonobese diabetic mice by dendritic cell transfer. J Clin Invest 90:741-748,1992
18. Majno G, Joris I, Handler E, Desemone J, Mordes JP, Rossini A. A pancreatic venular defect in the BB/Wor rat. Am J Pathol 128:210-215,1987
19. Heaton DA, Millward BA, Gray IP, Yun Y, Hales CN, Pyke DA, Leslie RDG. Increased proinsulin levels as an early indicator of beta-cell dysfunction in non-diabetic twins of type I (insulin-dependent) diabetic patients. Diabetologia 31:182-184, 1988
20. Hartling SG, Lindgren F, Dahlquist G, Persson B, Binder C. Elevated proinsulin in healthy siblings of IDDM patients independent of HLA identify. Diabetes 38:1271-1274,1989
21. Palmer JP, Helqvist S, Soinas GA, Molvig J, Mandrup-Poulsen T, Andersen H, Nerup J. Interaction of β-cell activity and exposure time in isolated rat islets of Langerhans. Diabetes 38:1211-1216,1989
22. Faustman D, Li X, Lin HY, Fu Y, Eisenbarth G, Guo J. Linkage of faulty Major Histocompatibility Complex class I to autoimmune diabetes. Science 254:1756-1761, 1991
23. Serreze DV, Leiter EH. Immunostimulation circumvents diabetes in NOD/Lt mice. J Autoimmunity 2:759-776,1989
24. Sempé P, Ezine S, Marvel J, et al. Role of $CD4^+CD45RA^+$ T cells in the development of autoimmune diabetes in the nonobese (NOD) mouse. Int Immunol 5:479-489,1993
25. Shehadeh NN, LaRosa F, Lafferty KJ. Altered cytokine activity in adjuvant inhibition of autoimmune diabetes. J Autoimmun 6:291-300,1993
26. Gosh S, Palmer SM, Rodrigues NR, Cordell HJ, Hearne CM, Cornall RJ, Prins JB, McShane P, Lathrop GM, Peterson LB. Polygenic control of autoimmune diabetes in nonobese diabetic mice. Nat Genet 4:404-409,1993
27. Krieg AM, Steinberg AD. Retroviruses and autoimmunity. J Autoimmun 3:137-166,1990
28. Wahl LM, Cocoran ML, Pyle SW, Arthur LO, Harel-Bellan A, Farrar WL. Human immunodeficiency virus glycoprotein (gp120) induction of monocyte arachidonic acid metabolites and interleukin 1. Proc Natl Acad Sci USA 86:621-625,1989
29. Gottlieb RA, Lennarz WJ, Knowles RD, Ciancolo GJ, Dinarello CA, Lachman LB, Kleinerman ES. Synthetic peptide corresponding to a conserved domain of the retroviral protein p15E blocks IL-1 mediated signal transduction. J Immunol 142:4321-4328,1989
30. Menser MA, Forrest JM, Bransby RD. Rubella infection and diabetes mellitus. Lancet 1:57-60,1978
31. Yoon JW. The role of viruses and environmental factors in the induction of diabetes. Curr Top Microbiol Immunol 164:95-124,1990
32. Williams LL, Shannon BT, Leguire LE, Fillman R. Persistently altered T cell immunity in high school students with the congenital rubella syndrome and profound hearing loss. Pediatr Infect Dis J 12:831-835,1993
33. Charlton B, Bacelj A, Slattery RM, Mandel TE. Cyclophosphamide-induced diabetes in NOD/WEHI mice. Evidence for suppression in spontaneous autoimmune diabetes mellitus. Diabetes 38:441-447,1989
34. Rossini AA, Appel MC, Like AA. Genetic influence of the streptozocin-induced insulitis and hyperglycemia. Diabetes 26:916-920,1977
35. Sakane T, Green I. Specificity and suppressor function of human T cell responses to autologous non-T cells. J Immunol 123:584-589,1979

36. Kantwerk-Funke G, Burkart V, Kolb H. Low dose streptozocin causes stimulation of the immune system and of anti-islet cytotoxicity in mice. Immunology 86:266-270,1991
37. Oldstone MBA, Southern P, Rodriguez M, Lampert P. Virus persists in β-cells of islets of Langerhans and is associated with chemical manifestations of diabetes. Science 224:1440-43,1984
38. Sairenji T, Diabata M, Sorli CH, Qvistbäck H, Humphreys RE, Ludvigsson J Palmer J, Landin-Olsen M, Sundkvist G, Michelsen B, Lernmark Å, Dyrberg T. Relating homology between Epstein-Barr virus BOLF1 molecule and HLA-DQw8 chain to recent onset type 1 (insulin-dependent) diabetes mellitus. Diabetologia 34:33-39,1991
39. Wilkin TJ. The primary lesion theory of autoimmunity: a speculative hypothesis. Autoimmunity 7:225-235, 1990
40. Kolb-Bachofen V, Epstein S, Kiesel U, Kolb H. Low-dose streptozocin-induced diabetes in mice. Electron microscopy reveals single cell insulitis before diabetes onset. Diabetes 37:21-27,1988
41. Muir A, Rovin BH, Lacy PE, Schreiner GF. Macrophage-specific chemotactic lipid release by in vivo streptozocin-administered mouse islets. Diabetes 40:1459-1466,1991
42. Oschelewski M, Schwab E, Kiesel U, Opitz U, Stünkel K, Kolb-Bachofen V, Kolb H. Administration of silica or monoclonal antibody to Thy-1 prevents low-dose streptozocin-induced diabetes in mice. Immunol Lett 12:289-295,1986
43. Palmer JP, Helqvist S, Spinas GA, Molvig J, Mandrup-Poulsen T, Andersen HU, Nerup J. Interaction of β-cell activity and IL-1 concentration and exposure time in isolated rat islets of Langerhans. Diabetes 38:1211-1216,1989
44. Yoon J-W, Austin M, Onoderea T, Notkins AL. Virus-induced diabetes mellitus. Isolation of a virus from the pancreas of a patient with diabetic ketoacidosis. N Engl J Med 300:1173-1179,1979
45. Gerling IC, Serreze DV, Christianson SW, Leiter EH. Intrathymic islet cell transplantation reduces β-cell autoimmunity and prevents diabetes in NOD/Lt mice. Diabetes 41:1672-1676,1992
46. Shehadeh N, Calcinaro F, Bradley BJ, Bruchlim I, Vardi P, Lafferty KJ. Effect of adjuvant therapy on development of diabetes in mouse and man. Lancet 343:706-707,1994
47. Bowman MA, Leiter EH, Atkinson MA. Prevention of diabetes in the NOD mouse: implications for therapeutic intervention in human disease. Immunology Today 15:115-120,1994
48. Tian J, Kamarzarian A, Lehmann PV, Atkinson M, Chen W, Clare-Salzler M, Kaufman D. Induced tolerance to peptides of glutamate decarboxylase prevents murine IDDM. Abstract presented on 13th international immunology and diabetes workshop,1994
49. Muir A, Cornelius J, Ramiya V, Krischner J, Peck A, MacLaren N. NOD mouse immunization to insulin β chain protects against diabetes and activates regulatory T lymphocytes thereby reducing intra-islet interferon (IFN)-γ. Abstract presented on 13th international immunology and diabetes workshop,1994
50. Keller R, Eisenbarth G, Jackson R. Insulin prophylaxis in individuals at high risk of type I diabetes. Lancet 341:927-928,1993

SUMMARY

Chapter 1: General introduction

Diabetes mellitus type 1 is the outcome of an autoimmune process resulting in β-cell loss. Genetic factors, especially genes influencing immune function, like the genes encoding for MHC-class II, are important in the development of type 1 diabetes. Also environmental factors (viruses, toxins) are likely to play role.
Animal models for type 1 diabetes mellitus are important tools in the investigation of the mechanisms leading to autoimmune mediated β-cell loss. One of the models used most frequently in diabetes research is the spontaneously diabetic NOD mouse, a model showing many similarities with the human disease. Research into this animal model made it clear that T-cells of both the $CD4^+$ and the $CD8^+$ subset play an essential role in the development of type 1 diabetes. Elimination of either one of these subsets prevents the development of diabetes. However, before such T-cells can mount a β-cell specific immune response, islet auto-antigens must be presented in the appropriate form and context to these T cells by specialized antigen presenting cells.
Three types of immune cells exert an antigen presenting function: dendritic cells, macrophages and B-cells. Dendritic cells are bone-marrow derived. Precursors for the so-called monocyte-derived dendritic cells circulate in the monocytic pool of the peripheral blood. Although dendritic cells are heterogeneous in their morphology, localization and function, all dendritic cells are capable of stimulating naive T-cells to an immune response. In this capacity dendritic cells are unique. Like dendritic cells, macrophages are bone-marrow derived cells that show a striking heterogeneity in morphology and function. Precursors for macrophages also circulate in the monocytic pool of the peripheral blood. It is not clear whether all dendritic cells and macrophages share a common precursor.
At the onset of and during the insulitis process dendritic cells and macrophages can be found around and in the islets of Langerhans. In vivo experiments in animal models confirmed the essential role of macrophages for the development of diabetes: elimination of macrophages prevents diabetes to develop. Disturbances in macrophage maturation and function have been described in the NOD mouse. With regard to the function of dendritic cells, it was surprisingly found that transfer of dendritic cells from the pancreas draining lymph node of diabetic mice was able to prevent development of diabetes in young pre-diabetic NOD-mice.

Considering the above, this thesis is focussed on a further detailed immunohistochemical and functional characterization of macrophages and dendritic cells involved in the development of type 1 diabetes. The immunohistochemical

characterization was performed both on human pancreases, and on the pancreases of NOD mice, either during the spontaneous development of disease or after various treatments, influencing the course of the disease. Functional capability of dendritic cells from type 1 diabetic patients to act as stimulator cells for T cells was assessed using monocyte-derived dendritic cells from these patients.

Chapter 2: An immunohistochemical study on organized lymphoid cell infiltrates in fetal and neonatal pancreases. A comparison with similar infiltrates found in the pancreas of a diabetic infant

In chapter 2 an immunohistochemical study of the presence of T-cells, B-cells, dendritic cells, and macrophages in normal fetal and neonatal pancreases is described. The results are compared to results obtained in the pancreas of an 8 months old child, who died shortly after clinical onset of diabetes.
The presence of a few isolated dendritic cells and T cells in the exocrine pancreas appeared to be a normal feature of the normal fetal and neonatal pancreas. Also larger focal leucocytic infiltrates were detected in these pancreases. These consisted mainly of areas of T cells, sometimes containing high endothelial venule (HEV)-like structures, and dendritic cells and macrophages. These focal leucocytic infiltrates were present in the connective tissue of septa and capsules of the pancreases.
In the pancreas of the diabetic child the infiltration by isolated dendritic cells and T cells was more pronounced. Focal leucocytic infiltrates were again found, but comprised a significantly larger percentage of the surface area of the sections, as compared to normal neonatal pancreases. These focal leucocytic infiltrates in the pancreas of the diabetic child were not confined to the connective tissue compartments, but spreaded in between the exocrine acini.
The function of the focal leucocytic infiltrates present in normal fetal and neonatal pancreases is unknown. It is discussed whether they represent accumulations of immune cells playing a role in tolerance induction towards islet cell antigens.

Chapter 3: Immunohistochemical studies on murine pancreases during spontaneous development of IDDM, and after various treatments accelerating or postponing the disease

Chapter 3.1: Immunohistochemical characterization of monocytes-macrophages and dendritic cells involved in the initiation of insulitis and β-cell destruction in NOD mice

The infiltration pattern of monocytes-macrophages and dendritic cells during the spontaneous development of insulitis and diabetes in the NOD mouse is described in chapter 3.1.
Accumulation of two types of cells (ER-MP23$^+$ dendritic-shaped cells and MOMA1$^+$ macrophages) around the islets, both likely involved in antigen presentation, was found preceding the T- and B-lymphocyte accumulation.
Infiltration *into* the islets already at young age (10 weeks) by a specific type of phagocytotic/cytotoxic macrophage (BM8$^+$ macrophage) was found to be associated with a high diabetes incidence at 30 weeks of age. Infiltration by BM8$^+$ macrophages into the islet mass may represent an infiltration meant to remove debris caused by the surrounding peri-insulitis, or may play a role in the actual destruction of the β-cells.

Chapter 3.2: Effect of prophylactic insulin treatment on the number of antigen presenting cells and macrophages in the pancreas of NOD mice:
Is the prevention of diabetes based on ß-cell rest?

Chapter 3.2 describes the effect of prophylactic insulin treatment in the NOD mouse on the islet morphology and on the infiltration-pattern of antigen-presenting cells in the pancreas and around the islets.
In normal untreated NODs, and also in NOD-*scid/scids*, islets are considerably larger than islets found in control BALB/c. Prophylactic insulin treatment with exogenous insulin was found to reduce the islet size of NODs to sizes comparable to BALB/c islets. This reduction in islet size was accompanied by a significantly lower infiltration of ER-MP23$^+$ antigen presenting cells into the pancreas and around the islets.
We like to explain these data as suggesting that there is an abnormal β-cell metabolism in the NOD that attracts high numbers of antigen presenting cells. Treatment with exogenous insulin downregulates this abnormal β-cell metabolism resulting in smaller islets and a lower attraction of antigen presenting cells, and hence a slower development of insulitis and diabetes.

Chapter 3.3: The diabetes retardant effect of intrathymic implantation of syngeneic islets in NOD/Lt mice is associated with reduced islet infiltration by phagocytotic macrophages. A preliminary report

Intrathymic injection of islet cells results in a significant delay in the onset of diabetes.

In chapter 3.3 a preliminary report on the effect of intrathymic injection of islet cells on the infiltration-pattern of phagocytotic/cytotoxic $BM8^+$ macrophages around and into the islets is described. After intrathymic injection of islet-cells, the infiltration of $BM8^+$ macrophages into the islets is significantly reduced, although there is a considerable peri-insulitis. The percentage of islets not affected by peri-insulitis was even significantly reduced after intrathymic injection of islet cells.
It is speculated that intrathymic injection of islet cells induces an altered cytokine milieu in the T-cell peri-insulitis process, which does not provide the signals necessary for $BM8^+$ macrophages to infiltrate. Another possibility is that the altered cytokine milieu results in a lower β-cell death, causing a reduced need for $BM8^+$ macrophages to infiltrate.

Chapter 3.4: Orchidectomy accelerates the progression of insulitis, focal pancreatitis, and sialoadenitis in male nonobese diabetic (NOD) mice. An immunohistopathological study on macrophage and lymphocyte infiltration

In chapter 3.4 the effect of gonadectomy on diabetes development, the progression of the insulitis process, and the development of focal leucocytic infiltrates in the pancreas and in the submandibular gland is described. It was found that orchidectomy in male NOD mice resulted in a faster progression to more severe forms of insulitis and an earlier ß-cell loss, reflected in a faster development of diabetes. There also was an enhancement of the formation of focal leucocytic infiltrates in the exocrine pancreas, and in the submandibular gland. Ovariectomy in female NOD mice had no effect. Orchidectomy did not have an effect on the cellular composition of the inflammatory processes, or on the order of immune events leading to ß-cell destruction.
It is discussed that an effect of androgens on the production of cytokines in these inflammatory processes may play a role in the observed acceleration of inflammatory processes in androgen deprived mice.

Chapter 4: Evidence for a defective maturation and function of antigen presenting cells in type 1 diabetics. A factor of importance in the disturbed tolerance towards β-cell antigens?

Disturbances in the maturation and function of monocyte-derived dendritic cells in type 1 diabetic patients are described in chapter 4.
Dendritic cells were either isolated from peripheral blood via density gradients, or were generated from peripheral blood monocytes of type 1 diabetic patients of varying age

and disease duration, including two patients not yet treated with insulin. Maturation from monocytes, capability of the dendritic cells to form cellular clusters, and the capacity to act as stimulator cells in a syngeneic or allogeneic MLR were tested.
It was found that a substantial proportion of the type 1 diabetic patients had a reduced capacity to mature dendritic cells from peripheral blood monocytes. Dendritic cells from type 1 diabetic patients also showed a reduced capability to form cellular clusters, and to act as stimulator cells in MLR. There was no relation of these disturbances with duration of the disease, presence of autoantibodies, sex, or age of the patients.
The lack of correlation with duration of the disease (disturbances were found even after 10-15 years after onset of diabetes) is suggestive of intrinsic abnormalities in dendritic cell maturation and function in type 1 diabetic patients. It is discussed that a defective maturation and function of dendritic cells may result in an inefficient antigen presentation, which is able to support a stimulation of effector T-cells in the periphery, but is unable to induce tolerance.

Chapter 5: General conclusions and discussion

From this thesis it can be concluded:
a) that the presence of a few antigen presenting cells in the exocrine pancreas and near the islets is a normal phenomenon,
b) that various subclasses of antigen presenting cells and macrophages are involved in the initiation of the insulitis and in the β-cell destruction,
c) that local factors like a disturbed β-cell metabolism and/or a vascular abnormality are associated with the attraction of high numbers of antigen presenting cells to the pancreas,
d) that antigen presenting cells are disturbed in their functions in a large proportion of type 1 diabetics,
e) that types of insulitis exist which are not β-cell destructive.
In chapter 5 also a hypothesis is formulated on the pathogenesis of type 1 diabetes, based on the involvement of both dendritic cells and macrophages in the process leading to type 1 diabetes, and on the disturbances in the function of these cells. In the initial phases of the autoimmune process high numbers of functionally abnormal antigen presenting dendritic cells and macrophages, that have a reduced capability to induce T cell tolerance, become attracted to the islets. This elevated attractivity is probably caused by a disturbed β-cell metabolism, leading to the release of chemoattractants for these cells. The extravasation of the leukocytes may also be facilitated by abnormalities in the vasculature seen in NODs, characterized by a high endothelium and an enlarged

peri-vascular area. As a consequence, specific T effector cells are generated towards islet cell antigens, which also accumulate around the islet. The cytokine micro-environment of this peri-insulitis is most likely important for the progression to β-cell destruction by macrophages, and for the development of diabetes due to specific β-cell loss.

The hypothesis assumes in fact two major disturbances. The first is a local disturbance on the level of the islet itself (e.g. an abnormal ß-cell metabolism), attracting large numbers of antigen presenting cells to the islet. The second is a systemic disturbance, namely a dysregulation of the immune reaction (a defective tolerance induction) due to an abnormal function of these attracted monocyte derived cells.

These two disturbances may be induced by genetic and/or environmental factors.

Prevention of diabetes may be possible by regimens that intend to correct either one (or both) of these disturbances.

SAMENVATTING

Hoofdstuk 1: Algemene inleiding

Diabetes mellitus type I is het eindresultaat van een autoimmuun proces, dat β-cel verlies tot gevolg heeft. Genetische factoren, en met name genen die de werking van het immuunsysteem beïnvloeden, zoals de genen voor MHC klasse 2, zijn belangrijk bij het ontwikkelen van type I diabetes. Ook omgevingsfactoren (virussen, toxische stoffen) spelen waarschijnlijk een rol.
Diermodellen voor type I diabetes zijn belangrijke instrumenten om de mechanismen die leiden tot autoimmuun gemedieerd β-cel verlies te kunnen onderzoeken. Een van de modellen, die het vaakst gebruikt wordt in diabetes onderzoek, is de NOD muis, die spontaan diabeet wordt. Dit model vertoont vele gelijkenissen met de ziekte bij de mens. Onderzoek aan dit model maakte duidelijk dat T-cellen van de $CD4^+$ en van de $CD8^+$ subklasse beide een essentiële rol spelen bij het ontstaan van diabetes. Wanneer een van deze subklassen geëlimineerd wordt zal diabetes niet ontstaan. Maar voordat zulke T-cellen een β-cel specifieke reactie kunnen vertonen, moet eiland-autoantigeen door gespecialiseerde antigeenpresenterende cellen worden gepresenteerd aan deze T-cellen in de geschikte vorm en context.
Drie celtypen voeren deze antigeen presenterende functie uit: dendritische cellen, macrofagen en B-cellen. Dendritische cellen komen uit het beenmerg. Voorlopers van de zogenaamde "van monocyt afkomstige dendritische cellen" circuleren in het monocytaire compartiment van het perifere bloed. Hoewel dendritische cellen heterogeen zijn in morfologie, lokalisatie en functie, kunnen alle dendritische cellen naïeve T-cellen aanzetten tot een immuun respons. Deze eigenschap is uniek voor dendritische cellen. Net als dendritische cellen komen macrofagen ook uit het beenmerg, en vertonen ook een opvallende heterogeniteit in morfologie en functie. Voorlopers van macrofagen circuleren ook in de monocytaire pool van het perifere bloed. Het is niet duidelijk of alle dendritische cellen en macrofagen uit dezelfde voorloper kunnen ontstaan.
Bij het ontstaan van, maar ook tijdens de insulitis, kunnen dendritische cellen en macrofagen rond en in de eilandjes worden aangetroffen. In vivo experimenten in diermodellen bevestigden de essentiële rol van macrofagen bij het ontstaan van diabetes: eliminatie van macrofagen voorkomt diabetes. Functie en maturatie stoornissen van macrofagen zijn beschreven in de NOD muis. Wat betreft de functie van dendritische cellen is, verrassend genoeg, gevonden dat overspuiten van dendritische cellen uit de pancreas-drainerende lymfklier het ontstaan van diabetes kan voorkomen in jonge NOD muizen.

Met het bovenstaande in gedachten, houdt dit proefschrift zich bezig met een verder

gedetailleerde immunohistochemische en functionele karakterisatie van macrofagen en dendritische cellen, die betrokken zijn bij het ontstaan van type I diabetes. De immunohistochemische karakterisatie is uitgevoerd op humane pancreassen en op pancreassen van NOD muizen, hetzij tijdens de spontane ontwikkeling van de ziekte, hetzij na diverse behandelingen die het verloop van de ziekte kunnen beïnvloeden. Voor het testen van het functionele vermogen van dendritische cellen van type I diabetische patiënten om als stimulator cel voor T-cellen te fungeren, werd gebruik gemaakt van monocyt-afkomstige dendritische cellen van deze patiënten.

Hoofdstuk 2: Een immunohistochemische studie van georganiseerde infiltraten van lymfoïde cellen in foetale en neonatale pancreassen. Een vergelijking met dergelijke infiltraten, die gevonden zijn in de pancreas van een diabetisch kind

In hoofdstuk 2 wordt een immunohistochemische studie beschreven van de aanwezigheid van T-cellen, B-cellen, dendritische cellen en macrofagen in normale foetale en neonatale pancreassen. De resultaten worden vergeleken met de resultaten die verkregen werden uit het bestuderen van de pancreas van een 8 maanden oud kind, dat kort na het klinische begin van diabetes was overleden.
De aanwezigheid van een klein aantal geïsoleerd voorkomende dendritische cellen en T-cellen is een normaal verschijnsel in normale foetale en neonatale pancreassen. Ook grotere focale leukocytaire infiltraten werden aangetroffen in deze pancreassen. Deze infiltraten bestonden voornamelijk uit T-cel gebieden, waarin soms hoog-endotheel-venule (HEV)-achtige structuren voorkwamen, en dendritische cellen en macrofagen. De focale leukocytaire infiltraten zaten in het bindweefsel van septa en kapsel van deze pancreassen.
In de pancreas van het diabetische kind was de infiltratie door geïsoleerde dendritische cellen en T-cellen meer uitgesproken. Focale leukocytaire infiltraten werden ook hier gevonden, maar besloegen een significant groter percentage van het oppervlak van de coupes dan in de normale neonatale pancreassen. Ook waren deze focale leukocytaire infiltraten niet beperkt tot de bindweefsel compartimenten, maar ze verspreidden zich tussen de exocriene acini.
De functie van de focale infiltraten in normale foetale en neonatale pancreassen is onbekend. Er wordt bediscussieerd of ze accumulaties van immuuncellen kunnen zijn, die een rol spelen in het induceren van tolerantie voor eilandcel antigenen.

Hoofdstuk 3: Immunohistochemische studies van muizepancreassen tijdens het spontane ontstaan van IDDM, en na verschillende behandelingen die het ontstaan van de ziekte versnellen of vertragen

Hoofdstuk 3.1: Immunohistochemische karakterizatie van monocyten-macrofagen en dendritische cellen die betrokken zijn bij de initiatie van insulitis en bij β-cel destructie in de NOD muis.

De infiltratiepatronen van monocyten-macrofagen en dendritische cellen tijdens het spontane ontstaan van insulitis en diabetes in de NOD muis worden beschreven in hoofdstuk 3.1.
Accumulatie van twee typen cellen rondom de eilandjes (ER-MP23$^+$ cellen met een dendritische morfologie en MOMA1$^+$ macrofagen), beide waarschijnlijk betrokken bij antigeen presentatie, wordt gevonden voordat T- en B-cellen aangetroffen kunnen worden bij de eilandjes.
Infiltratie op jonge leeftijd (10 weken) *in* de eilandjes door een specifiek type fagocyterende/cytotoxische macrofaag (BM8$^+$ macrofaag) was geassocieerd met een hoge diabetes incidentie op de leeftijd van 30 weken. De bedoeling van deze infiltratie door BM8$^+$ macrofagen zou het opruimen van debris, veroorzaakt door de omringende peri-insulitis, kunnen zijn, maar het zou ook een rol kunnen spelen bij de echte β-cel destructie.

Hoofdstuk 3.2: Effect van profylactische insuline behandeling op het aantal antigeen-presenterende cellen en macrofagen in de pancreas van de NOD muis: Is de preventie van diabetes gebaseerd op β-cel rust?

In hoofdstuk 3.2 wordt het effect van profylactische insuline behandeling in de NOD muis op de eiland morfologie en op het infiltratiepatroon van antigeenpresenterende cellen in de pancreas en rondom de eilanden beschreven.
In normale, onbehandelde NOD muizen, maar ook in NOD-*scid/scids* zijn eilandjes aanzienlijk groter dan de eilandjes in controle BALB/c muizen. Profylactische insuline behandeling met exogeen insuline bleek de eilandgrootte in NODs te reduceren tot groottes die vergelijkbaar zijn met BALB/c eilandjes. Deze reductie in eilandgrootte ging gepaard met een significante verlaging van het aantal in de pancreas en rond de eilandjes geïnfiltreerde ER-MP23$^+$ antigeenpresenterende cellen.
Wij leggen deze data graag uit als suggestief voor een abnormaal β-cel metabolisme, dat hoge aantallen antigeenpresenterende cellen aantrekt. Behandeling met exogeen insuline reguleert dit abnormale β-cel metabolisme naar beneden, wat resulteert in kleinere

eilandjes en een verminderd aantrekken van antigeenpresenterende cellen, waardoor een langzamer ontwikkeling van diabetes veroorzaakt wordt.

Hoofdstuk 3.3: De beschermende peri-insulitis die veroorzaakt wordt door intrathymale transplantatie van syngene eilandjes in NOD muizen wordt gekarakteriseerd door een verminderde infiltratie van fagocyterende macrofagen. Een voorlopig rapport.

Intrathymale injectie van eilandcellen heeft een significant later ontstaan van diabetes tot gevolg. In hoofdstuk 3.3 wordt een voorlopig rapport beschreven over het effect van intrathymale injectie van eilandcellen op het infiltratiepatroon van fagocyterende/cytotoxische $BM8^+$ macrofagen rondom en in de eilandjes. De infiltratie door $BM8^+$ macrofagen in de eilandjes is significant verlaagd na intrathymale injectie van eilandcellen, hoewel er nog steeds een aanzienlijke peri-insulitis is. Het percentage eilandjes dat niet aangedaan was door peri-insulitis, was zelfs significant lager na intrathymale injectie van eilandcellen.
Er wordt gespeculeerd dat intrathymale injectie van eilandcellen het cytokine-milieu in de T-cel peri-insulitis verandert, zodat deze niet meer voorziet in de signalen die nodig zijn voor $BM8^+$ macrofagen om te infiltreren in de eilandjes. Een andere mogelijkheid zou zijn dat het veranderde cytokine-milieu een verminderde β-cel dood tot gevolg heeft, waardoor $BM8^+$ macrofagen minder hoeven te infiltreren.

Hoofdstuk 3.4: Orchidectomie versnelt de progressie van insulitis, focale pancreatitis en sialoadenitis in mannelijke NOD muizen. Een immunohistopathologische studie van macrofagen- en lymfocyten-infiltratie.

In hoofdstuk 3.4 wordt het effect van gonadectomie op het ontstaan van diabetes, op de progressie van het insulitisproces en op de ontwikkeling van focale leukocytaire infiltraten in de pancreas en in de submandibulaire speekselklier beschreven. Gevonden is dat orchidectomie in mannelijke NOD muizen resulteert in een snellere progressie naar ernstiger vormen van insulitis en een eerder β-cel verlies, wat zich weerspiegelt in een eerder ontstaan van diabetes. Er werd ook een versnelling in de vorming van focale leukocytaire infiltraten in de exocriene pancreas en in de submandibulaire speekselklier gevonden. Ovariectomie in vrouwelijke NOD muizen had geen effect. Orchidectomie had geen invloed op de samenstelling van de ontstekingsprocessen, en ook niet op de volgorde van de immunologische gebeurtenissen die leiden tot β-cel vernietiging.
Er wordt besproken dat androgenen de produktie van cytokinen kunnen beïnvloeden, wat een rol zou kunnen spelen in de gevonden versnelling van de ontstekingsprocessen in androgeen-gedepriveerde dieren.

Hoofdstuk 4: Bewijs voor een gestoorde uitrijping en functie van antigeenpresenterende cellen in type I diabeten. Een belangrijke factor in de gestoorde tolerantie voor β-cel antigenen?

Stoornissen in de uitrijping en functie van van monocyten afkomstige dendritische cellen in type I diabetische patiënten worden beschreven in hoofdstuk 4.
Dendritische cellen werden ofwel geïsoleerd uit het perifere bloed met behulp van dichtheidsgradienten, of werden gerijpt uit monocyten uit het perifere bloed van type I diabetische patiënten van verschillende leeftijd en met een uiteenlopende ziekteduur, waaronder ook twee patiënten die nog niet behandeld waren met insuline. De uitrijping van monocyten, de mogelijkheid van de dendritische cellen om cellulaire clusters te vormen, en de capaciteit om als stimulatorcel te fungeren in een syngene of allogene gemengde lymphocyten kweek (MLR) werden getest.
Er werd gevonden, dat een belangrijk deel van de type I diabetische patiënten een verminderd vermogen had om dendritische cellen uit perifere-bloed-monocyten te laten uitrijpen. Dendritische cellen van type I diabeten vertoonden ook een verminderd vermogen om cellulaire clusters te vormen en om als stimulatorcel in een MLR te fungeren. Er werd geen relatie gevonden tussen deze stoornissen en de duur van de ziekte, de aanwezigheid van autoantistoffen, het geslacht of de leeftijd van de patiënten.
De afwezigheid van een correlatie met de duur van de ziekte (stoornissen konden zelfs 10-15 jaar na het begin van de ziekte nog aangetoond worden) is suggestief voor intrinsieke stoornissen in de maturatie en functie van dendritische cellen van type 1 diabetische patiënten. Er wordt besproken dat een gestoorde uitrijping en functie van dendritische cellen kan resulteren in een inefficiënte antigeenpresentatie, die nog wel de stimulatie van effector T-cellen in de periferie kan verzorgen, maar geen tolerantie kan induceren.

Hoofdstuk 5: Algemene conclusies en discussie

Uit dit proefschrift kan worden geconcludeerd:
a) dat de aanwezigheid van enkele antigeenpresenterende cellen in de exocrine pancreas en rondom de eilandjes een normaal verschijnsel is,
b) dat verschillende subklassen van antigeenpresenterende cellen en macrofagen betrokken zijn bij de initiatie van insulitis en bij de β-cel vernietiging,
c) dat lokale factoren, zoals een gestoord β-cel metabolisme en/of een vasculaire afwijking geassocieerd zijn met het aantrekken van hoge aantallen

antigeenpresenterende cellen naar de pancreas,
d) dat in een groot aantal type 1 diabetische patiënten de functie van antigeenpresenterende cellen gestoord is,
e) dat er vormen van insulitis bestaan die niet β-cel destructief zijn.
In hoofdstuk 5 wordt ook een hypothese geformuleerd over de pathogenese van type I diabetes, die gebaseerd is op de gevonden betrokkenheid van dendritische cellen en macrofagen bij het ontstaan van diabetes, en op de funktiestoornissen die gevonden zijn in deze cellen. In de beginfase van het autoimuunproces worden grote aantallen afwijkende antigeenpresenterende dendritische cellen en macrofagen, die een verminderd vermogen hebben tot het induceren van T cel tolerantie, aangetrokken naar de eilandjes. Deze verhoogde aantrekkingskracht wordt waarschijnlijk veroorzaakt door een gestoord β-cel metabolisme, dat leidt tot het vrijkomen van chemo-attractantia voor deze cellen. De extravasatie van deze leukocyten wordt mogelijk nog vergemakkelijkt door de vaatafwijkingen die gevonden worden in NODs, bestaande uit een verhoogd endotheel en een vergrote peri-vasculaire ruimte. Als gevolg van dit alles, worden effector T cellen gegenereerd, die specifiek zijn voor eiland-antigenen. Deze T-cellen accumuleren ook rond de eilandjes. Het cytokinemilieu van deze peri-insulitis is waarschijnlijk belangrijk voor de progressie naar β-cel destructie door macrofagen en voor het ontstaan van diabetes.
Deze hypothese gaat er van uit, dat er twee belangrijke stoornissen bestaan. De eerste is een lokale stoornis op het niveau van het eilandje zelf (een afwijkend β-cel metabolisme), die grote aantallen antigeenpresenterende cellen aantrekt tot het eilandje. De tweede is een systemische stoornis, namelijk een dysregulatie van de immuunreactie (een gestoorde tolerantie-inductie), als gevolg van een gestoorde functie van deze van monocyten afkomstige cellen.
Deze twee stoornissen kunnen het gevolg zijn van zowel genetische als omgevingsfactoren.
Preventie van diabetes zou mogelijk kunnen zijn met behulp van programma's, die gericht zijn op het corrigeren van een van deze (of beide) stoornissen.

Toelichting

Dit hoofdstuk is toegevoegd aan het eigenlijke proefschrift, om mensen, die geïnteresseerd zijn in het hoe en waarom van dit onderzoek, maar die niet voldoende op de hoogte zijn van de biologische en immunologische theorieën hierachter, uit te leggen waar dit boekje over gaat. Ik heb geprobeerd het voor iedereen begrijpelijk te houden. Om dit hoofdstuk echter leesbaar en beknopt te houden, heb ik een basiskennis van de biologie op middelbare-school-niveau aanwezig moeten veronderstellen.

Diabetes mellitus type 1

Diabetes mellitus, oftewel suikerziekte, is een ziekte die ontstaat door een tekort aan insuline, een hormoon dat in de alvleesklier aangemaakt wordt, wanneer de suikerspiegel van het bloed stijgt. De alvleesklier bevat hiertoe speciale cellen, de zogenaamde ß-cellen in de eilandjes van Langerhans.
Alle cellen in het lichaam hebben insuline nodig om glucose (suiker) uit het bloed op te kunnen nemen, zodat ze het als brandstof kunnen gebruiken. Bij een tekort aan insuline kunnen cellen het glucose niet meer op nemen, en ontstaat er een toestand waarin cellen een tekort aan brandstof hebben, terwijl er in het bloed veel te veel suiker aanwezig is. Dit tekort aan insuline heet suikerziekte.

Er zijn twee verschillende soorten suikerziekte. De meest voorkomende vorm is ouderdomssuikerziekte, oftewel diabetes mellitus type 2, die, zoals de naam al zegt, meestal op oudere leeftijd ontstaat.
De andere vorm is jeugdsuikerziekte, of diabetes mellitus type 1, die meestal in de kinderjaren of in de vroege volwassenheid ontstaat. Het onderscheid tussen deze twee typen wordt gemaakt, omdat het mechanisme waardoor ze veroorzaakt worden verschillend is.

Dit proefschrift gaat over jeugdsuikerziekte.
Bij jeugdsuikerziekte bevatten de eilandjes van Langerhans geen ß-cellen meer. Hierdoor is er geen insulineproduktie meer mogelijk. Mensen met deze aandoening moeten dus insuline van buitenaf toegediend krijgen: spuiten. (Overigens moeten veel patiënten met ouderdomsdiabetes ook insuline spuiten.)
Eind 60'er-begin 70'er jaren werd gevonden hoe het kwam dat er geen ß-cellen meer in de eilandjes van Langerhans van type 1 diabeten aanwezig waren:
het immuunsysteem had de ß-cellen vernietigd.
Waarom dat gebeurt is een vraag die de wetenschap nog steeds bezig houdt. Het onderzoek dat beschreven is in dit boekje, probeert een antwoord op deze vraag te geven.
Om uit te kunnen leggen waar dit boekje precies over gaat, moet ik eerst de werking van het immuunsysteem nader toelichten.

Het immuunsysteem

Dagelijks komen wij in contact met duizenden ziekteverwekkers, zoals bacteriën, virussen en parasieten. Dat wij niet van al deze ziekteverwekkers ziek worden komt door

de bescherming die onze afweer ons biedt.
Ten eerste hebben we natuurlijk onze huid en onze slijmvliezen, die, zolang ze intact zijn, een goede mechanische barrière vormen tegen al deze ziektekiemen. Komen ziektekiemen toch door deze barrière dan zal ons immuunsysteem in de meeste gevallen die ziekteverwekkers herkennen en onschadelijk maken. Om dit efficiënt te kunnen doen, bestaat het immuunsysteem uit verschillende typen cellen met allemaal hun eigen takenpakket. De meeste van deze cellen worden aangemaakt in het beenmerg, maar ondervinden vaak nadat ze het beenmerg verlaten hebben nog een soort nascholing, waardoor ze hun taken beter kunnen uitvoeren.
De cellen van het immuunsysteem verlaten het beenmerg via het bloed, en zijn in het bloed beter bekend als "witte bloedcellen". Nadat ze korte of langere tijd in het bloed gecirculeerd hebben, verlaten de witte bloedcellen de bloedbaan via de vaatwand, op een plaats waar het bloed langzaam stroomt en het vat dun is: in de haarvaten of vlak na de haarvaten. Ze komen dan terecht in de weefsels van de verschillende organen. Zo bevinden de cellen van het immuunsysteem zich in bijna het gehele lichaam. Ze surveilleren in de weefsels op zoek naar dingen die er niet thuis horen. Het immuunsysteem moet dus op de een of andere manier een onderscheid kunnen maken tussen wat van het lichaam zelf is (eigen), en wat dat niet is (vreemd).
Dit onderscheid wordt gemaakt via gespecialiseerde cellen, zogenaamde *antigeenpresenterende cellen (APC)*. Deze cellen onderzoeken alles wat ze in het lichaam tegen komen. Kleine fragmentjes van structuren op cellen, bacteriën, enz. (antigenen) worden op de buitenkant van deze cellen aan de overige cellen van het immuunsysteem gepresenteerd. De overige cellen van het immuunsysteem reageren op deze antigeenpresentatie met hetzij een *afweerreactie*, hetzij juist een geheel stil leggen van hun activiteit voor kortere of langere duur, *tolerantie*. Hoe deze reactie uitvalt hangt onder andere ervan af, of gelijktijdig een label herkend wordt, dat aanwezig is op eigen cellen, maar niet op indringers. Ieder mens heeft zijn eigen (bijna) unieke label op zijn cellen zitten, de transplantatie antigenen. Verder wordt de aard van de reactie op gepresenteerde antigenen waarschijnlijk bepaald door de signalen die de APC tijdens het presenteren uitzendt.

Als alles goed gaat wordt alleen "vreemd" opgeruimd, en stopt de afweerreactie, zodra deze als vreemd herkende structuren niet meer aanwezig zijn. De rommel die ontstaat na het vernietigen van vreemde structuren wordt in het algemeen opgeruimd door zogenaamde *macrofagen*, letterlijk vertaald: grote eters. Verder kunnen macrofagen ook zelf bacteriën of vreemde cellen doden door stofjes in hun omgeving los te laten, die deze cellen of bacteriën kapot maken.

In principe reageert het immuunsysteem dus op vreemd en laat het eigen met rust. Bij jeugdsuikerziekte is er iets misgegaan. Bij kinderen die net suikerziekte hebben gekregen

zie je namelijk een afweerreactie in en rondom de eilandjes van Langerhans. Als de afweerreactie is afgelopen zijn de ß-cellen uit de eilandjes verdwenen. Waarschijnlijk worden de ß-cellen per ongeluk als vreemd herkend, aangevallen en vernietigd.

Hoe kan dat?

Proefdieren

Wanneer mensen type 1 suikerziekte krijgen zijn bijna alle ß-cellen in de eilandjes al dood. Als je wilt weten waarom het immuunsysteem de ß-cellen niet als eigen heeft herkent zal je helemaal aan het begin van de afweerreactie moeten kijken wat er mis ging in de herkenning. Hier ligt een probleem: het is moeilijk te voorspellen wie er suikerziekte gaat krijgen, en wie niet. Alleen in families waarin suikerziekte voorkomt is deze voorspelling redelijk goed mogelijk, maar ook dan pas wanneer de vernietiging van ß-cellen al een heel eind gevorderd is. Wil je helemaal aan het begin kunnen onderzoeken wat er gebeurt, dan heb je dus een situatie nodig die goed voorspelbaar is. Daarom zijn er proefdieren ontwikkeld, waarvan je weet dat ze later suikerziekte zullen krijgen. Je hoopt dan dat deze proefdieren voldoende op de mens lijken om de vergelijking met suikerziekte bij mensen te kunnen maken. Eén van deze proefdieren is de nonobese diabetic (NOD) muis, die is gebruikt voor een aantal van de onderzoeken die in dit proefschrift beschreven zijn.

Afwijkingen in proefdieren die type 1 suikerziekte krijgen

We hebben gevonden dat in de alvleesklier van NOD muizen, vlak bij de eilandjes van Langerhans, hoge aantallen antigeenpresenterende cellen (APC), de cellen van het immuunsysteem die antigeen aan andere cellen presenteren, aanwezig zijn. Veel hogere aantallen dan je normaliter vindt in de alvleesklier van gewone muizen, die geen suikerziekte krijgen.

Aan de eilandjes zelf wordt ook iets geks gezien: ze zijn veel groter dan de eilandjes van gewone muizen.

Wanneer je nu NOD muizen vanaf heel jonge leeftijd, dus voordat ze suikerziekte hebben, gaat inspuiten met insuline, dan zie je dat een groot deel van deze muizen geen suikerziekte krijgt, of dat het moment waarop ze suikerziekte krijgen veel later is dan wanneer je ze niet zou hebben behandeld.
Kijk je tijdens deze behandeling naar de eilandjes van Langerhans, dan zie je dat de grootte van de eilandjes in deze behandelde muizen vergelijkbaar is met de grootte van eilandjes in gewone muizen, die geen suikerziekte krijgen. Bovendien zie je dat het

aantal APC in de alvleesklier van deze met insuline behandelde NOD muizen belangrijk afgenomen is.
Het lijkt er dus op dat er iets in de eilandjes van Langerhans van NOD muizen is, dat APC aantrekt. Wanneer je NOD muizen inspuit met insuline, waardoor ze zelf minder insuline hoeven te maken, trekken de eilandjes geen grote aantallen APC meer aan.

Nadat de APC zich verzameld hebben in de buurt van de eilandjes, komen er ook andere cellen van het immuunsysteem naar de eilandjes toe. Op een gegeven moment zie je bepaalde macrofagen de eilandjes in gaan. Pas nadat deze macrofagen de eilandjes in zijn gegaan, kun je grote hoeveelheden dode ß-cellen in de eilandjes aantonen. Of de macrofagen naar binnen gaan om dode ß-cellen op te ruimen, danwel om ze kapot te maken is niet duidelijk. Het is ook mogelijk dat er in eerste instantie macrofagen naar binnen gaan om dode ß-cellen op te ruimen, en dat er daarbij per ongeluk ook andere ß-cellen gedood worden, etc.

Hierboven werd beschreven, dat APC zich verzamelen in de buurt van de eilandjes van Langerhans. Dat ze zich verzamelen werd geassocieerd met het feit dat de eilandjes er anders uit zien. Maar waarom zetten deze APC het immuunsysteem aan tot een afweerreactie tegen iets van het eigen lichaam? De eilandjes mogen dan wel anders zijn dan gewone eilandjes, maar het immuunsysteem kent toch alleen deze eilandjes, en zou ze toch als eigen moeten herkennen?

Daarover gaan hoofdstuk vier en vijf.

Afwijkingen in mensen met type 1 suikerziekte
Als je bij mensen jonge cellen uit het bloed haalt die voorlopers zijn van dendritische cellen, een speciaal soort APC, dan kun je daar met bepaalde chemische stoffen dendritische cellen van maken in een reageerbuisje.
Probeer je datzelfde met jonge cellen van type 1 diabeten dan gaat dat veel minder goed: je krijgt minder grote aantallen dendritische cellen (DC) uit het zelfde aantal jonge voorlopers. Bovendien zijn de DC die je nog wel hebt kunnen maken minder actief: ze zijn minder goed in staat cellen van het immuunsysteem aan te zetten tot een reactie. Het lukt vaak nog wel, maar het gaat veel minder efficiënt dan in mensen zonder type 1 suikerziekte.

Een theorie
Als je in wetenschappelijke tijdschriften leest wat andere mensen hebben onderzocht aan het immuunsysteem, dan vind je de laatste tijd steeds meer aanwijzingen dat het

zodanig besturen van cellen van het immuunsysteem, dat geen reactie op "eigen" wordt opgewekt, het oproepen van tolerantie, veel meer moeite kost van APC, dan het opwekken van een afweerreactie.
Als het inderdaad zo is dat het oproepen van tolerantie veel meer moeite kost dan het opwekken van een afweerreactie, en DC van type 1 diabeten zijn minder efficiënt in het opwekken van een reactie van de overige cellen van het immuunsysteem, dan zou het zo kunnen zijn dat DC van mensen met type 1 suikerziekte nog wel een afweerreactie kunnen opwekken, maar dat het oproepen van tolerantie minder goed verloopt.

Met de hierboven beschreven onderzoeken kan dan de volgende theorie worden opgesteld:
Afwijkingen aan de eilandjes van Langerhans trekken abnormaal grote hoeveelheden APC aan. Deze APC hebben een licht gestoorde functie, waardoor ze nog wel een afweerreactie kunnen opwekken, maar het oproepen van tolerantie minder goed verloopt. Zo krijg je in de buurt van de eilandjes van Langerhans een groot aantal APC die de neiging hebben aan te sturen op een afweerreactie. De afweerreactie komt op gang tegen de ß-cellen in deze eilandjes, waardoor de ß-cellen worden vernietigd. Als bijna alle ß-cellen dood zijn, kan het lichaam niet voldoende insuline meer maken en ontstaan de symptomen van suikerziekte.

Dankwoord

Zonder de hulp en steun van velen was dit boekje niet geschreven. Hoewel ik niet iedereen op deze plaats kan noemen, wil ik enkelen hier toch persoonlijk bedanken.

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Herbert, mijn co-promotor. Hoewel je officieel mijn begeleider was, was met name het feit dat ik niet op jouw lab zat er de reden van dat je me niet zo veel gezien hebt. Toch stond je altijd voor me klaar, als ik problemen had, hoe druk je het ook had. En je kwam ook altijd even terug op een manuscript, dat ik op je bureau gelegd had. Jij bent iemand die complimentjes uitdeelt als er iets goed gegaan is. Misschien weet je niet eens hoe zeer ik het nodig had, om af en toe te horen dat ik goed werk afgeleverd had. Het gaf me steeds de opkikker die ik nodig had om vol te houden.

Rob, als hoofd van de afdeling was je steeds op de achtergrond aanwezig. Hoewel je je niet direkt met het onderzoek hebt bemoeid, wist je wel precies wat er speelde. En dat niet alleen wat het onderzoek betreft, maar ook je belangstelling voor persoonlijke zaken liet je duidelijk merken. Met name de keer dat je direkt enthousiast naar me toekwam toen je gehoord had dat mij een opleidingsplaats tot internist in Utrecht toegezegd was, staat me nog helder voor de geest.

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manuscripten, brieven, posters, abstracts en al het andere werk wat ik bij je op het bureau legde.

Joop, jij was 's morgens vaak de eerste die ik tegenkwam op de gang of bij de koffiepot. Omdat onze NOD muizen niet onder jouw hoede gefokt werden, heb ik niet zo veel met je te maken gehad als anders het geval zou zijn geweest, maar de paar controle muizen die ik toch nog nodig had wist je altijd nog wel op zeer korte termijn voor mij te regelen, zonder dat ik ze hoefde te bestellen.

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Pieter, jij was degene die me inwerkte. Later hadden we geregeld lange discussies over van alles en nog wat. Jouw verhalen over de verre landen die je bezocht had, en jouw visie op effecten van ontwikkelingshulp en westerse gezondheidszorg in niet-westerse landen waren een eye-opener voor me.

Peter, van jou heb ik leren kleuren. Toen ik dat eenmaal dacht te kunnen, liep ik natuurlijk nog geregeld tegen technische moeilijkheden op. Gelukkig nam je altijd de tijd om met me mee te denken over oplossingen. Jouw ervaring in kleurtechnieken is onmisbaar geweest voor de voortgang van mijn onderzoek.

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Jeroen and Herbert, I'll write this in English for Herbert. You both came almost at the same time and left almost at the same time, just before we had to move to the other side of the building. As soon as you were settled and accustomed to us you started to tease everybody. This made the atmosphere in the lab very lively. Or was it

because the lab was very crowded at that time? Anyway, I enjoyed being at the lab when you were there.

Belinda, de eerste student die ik meemaakte op het lab, geloof ik. Jij maakte bijna nog meer lawaai dan ik, en dat kon ik toch niet over m'n kant laten gaan. Tenslotte had ik de naam. Maar ik vond het wel hartstikke leuk dat je een tijdje bij ons gezeten hebt. Veel succes met je eigen onderzoek.

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Dear Martha, thank you for all the talks we had, especially in the evenings and late afternoons, when everybody had already gone home. It is not so bad having to work late, when there is somebody else too. I'm sorry, I can't really help you now that you're the only aio left in the lab. I hope there will be a new aio soon. But if you need somebody to talk to, you can always call me for a "borrel" at Dizzy's.

Lieve Maarten, jij bent degene die heeft gezorgd dat ik doorgezet heb toen ik het helemaal niet meer zag zitten. Als jij me er niet doorheen gepraat had, en toen al niet ervan overtuigd geweest was dat ik wel degelijk onderzoek kon doen, was dit boekje er nooit gekomen.

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Verder zijn er natuurlijk op de afdeling Immunologie zo'n honderd andere mensen met wie ik te maken heb gehad, en die mij vaak op een of andere manier geholpen hebben met het verwerken van materiaal, het verkrijgen van chemicaliën of monoclonalen of met adviezen. Op deze plaats wil ik iedereen daarvoor bedanken. Een paar mensen wil ik met name noemen.

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Dear Ed, the visit to your lab was a great impulse to my research. You may not even have noticed it, but, though it was only a short time, you taught me the scientific way of thinking: how to work from the results you get. In short: how does it fit into the facts you already have, what new viewpoints did arise, and what further experiments should be done. Before, I never worked so systematically with new results. Your way of thinking helped me a lot in managing my own research when I was back home again.

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