Fatal Attraction

Interactions between antigen-presenting cells and islets of Langerhans in the pathogenesis of autoimmune diabetes

Fatale aantrekkingskracht

Interacties tussen antigeen-presenterende cellen en eilanden van Langerhans in de pathogenese van autoimmuun diabetes

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Judith Gerarda Maria Rosmalen

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J'ai alors beaucoup réfléchi sur les aventures de la jungle et, à mon tour, j'ai réussi, avec un crayon de couleur, à tracer mon premier dessin.

Mon dessin numéro 1. Il était comme ça:

J'ai montré mon chef-d'oeuvre aux grandes personnes et je leur ai demandé si mon dessin leur faisait peur. Elles m'ont répondu: <<Pourquoi un chapeau ferait-il peur?>> Mon dessin ne représentait pas un chapeau. Il représentait un serpent boa qui digérait un éléphant. J'ai alors dessiné l'interieur du serpent boa, afin que les grandes personnes puissent comprendre. Elles ont toujours besoin d'explications.

Mon dessin numéro 2 était comme ça:

Le Petit Prince, Antoine de Saint-Exupéry

Aan mijn ouders

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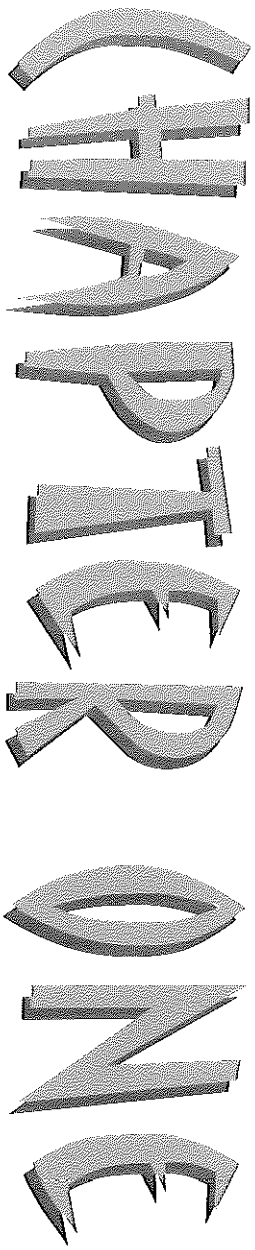
Preface

The onset of diabetes mellitus is characterized by various symptoms, all the result of a disturbed glucose metabolism. The main symptoms are thirst and an excessive production of urine. The disturbed glucose metabolism underlying these symptoms is due to an absolute deficiency of insulin secretion (type 1 diabetes mellitus), a reduction in its biological effectiveness (type 2 diabetes mellitus) or a combination of these factors. Type 1 diabetes mellitus is predominantly manifesting in children, and needs to be treated by life-long exogenous insulin administration to prevent high blood glucose levels. Type 2 diabetes mellitus occurs classically in adults, and is relatively milder in its appearance; usually, exogenous insulin administration is not required. This thesis concerns type 1 (insulin-dependent) diabetes mellitus (type 1 diabetes).

In the Netherlands, the annual incidence of type 1 diabetes among children younger than 14 years was 14.3 per 100,000 in the period from 1993-1995. Compared to earlier studies, the incidence has increased and the clinical manifestation has shifted to younger ages. Although the possibility of exogenous insulin administration has largely reduced the risk for short-term complications, such as diabetic ketoacidosis and hyperglycaemic coma, over 70% of patients will develop long-term complications. These include microangiopathy, that may cause problems with the kidney-function and the vision, and macropangiopathy, that leads to an elevated risk of developing major cardiovascular problems. The quality of life of diabetes patients can be profoundly reduced because of these complications. In addition, the life-expectancy of diabetes patients is considerably shorter than that of healthy persons. The risk of developing long-term complications may be significantly reduced by normalizing blood glucose levels. However, this requires intensive individual treatment and significant changes in lifestyle. Consequently, the disease a has large emotional and social impact on the lifes of patients and their family. In addition, diabetes has serious economic consequences, because of the costs of the treatment and lost productivity due to hospital admission.

Therefore, prevention and cure of the disease are major goals of diabetes research. In order to achieve these goals, a thorough knowledge is required about the mechanisms via which diabetes develops. The studies described in this thesis describe such a mechanism.



Diabetes pathogenesis in humans, BB rats and NOD mice: an overview

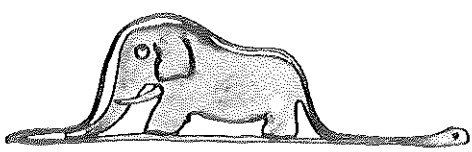
1.1 Destination diabetes: routes and detours in humans, BB rats and NOD mice.
Submitted

1.2 T cell education during diabetes development in humans, BB rats and NOD mice: teachers and students.
Submitted

1.3 Aim and outline of this thesis.

Chapter 1.1

Destination type 1 diabetes: routes and detours in humans, BB rats and NOD mice



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The ancient Egyptians already recognized the key finding of the disease we now know as diabetes mellitus: glucose intolerance. The first diagnostic test for this disease involved counting the number of bees attracted to a patient's urine, indicating the excess presence of glucose. The pancreatic nature of the disease was not discovered until 1889, when a research assistant noted that flies were especially attracted to urine of dogs without a pancreas. In 1921, the discovery of insulin explained this observation, a discovery that was rewarded with the Nobel Prize for physiology in 1923 for Banting and Macleod. With the discovery of insulin, it also became clear that diabetes is a chronic disease needing life-long treatment. In its various forms, diabetes affects about 5% of the population in most western societies. Today, two major types of diabetes mellitus are recognized, designated type 1 and type 2 diabetes. Type 1 diabetes mellitus is caused by an absence of insulin, whereas type 2 diabetes mellitus is related to an insensitivity for insulin. This review will focus on type 1 (insulin-dependent) diabetes mellitus (type 1 diabetes).

Symptoms of type 1 diabetes result from an absolute lack of insulin due to an autoimmune destruction of the β cells in the pancreatic islets of Langerhans. At least two observations indicate that autoimmunity is initiated long before clinical manifestation of diabetes. First, both T cell reactivity and autoantibodies against β cells can be detected several years before clinical onset in humans (1-3). Second, changes in the insulin response to glucose challenge may occur long before clinical onset (2). Several autoantigens appear to be involved in the autoimmune destruction (4-6). The absence of insulin results in hyperglycemia and severe catabolism with release of free fatty acids from adipose tissue and hepatic overproduction of ketones. Unless they are given exogenous insulin, affected individuals progress to excessive weight loss, ketoacidosis and dehydration and will finally die.

The causes of type 1 diabetes are largely unknown. The concordance in identical twins varies from 25-50% (7,8), indicating that both environmental and genetic factors are involved. In addition, there is a large difference in diabetes incidence between countries, probably related to genetic variations, as well as influence of temperature, differences in diet and hygienic circumstances (9,10). Among the genetic factors related to diabetes, the strongest genetic association is with the Major Histocompatibility Complex (MHC) (11,12), but also other immune function genes appear to be involved (13). It is thus not surprising that several other autoimmune diseases have been described to be associated with type 1 diabetes (14-18). In contrast to most other autoimmune diseases, the incidence in males and females is similar overall, but dependent on the subpopulation the ratio varies from 0.3-2.1 (18). The clinical features of type 1 diabetes development are summarized in Table 1.

Table 1: Clinical features of diabetes in NOD mice, BB rats and humans.

	NOD mouse	BB rat	Human
Lean-body habitus	+	+	+
Sex distribution	F>M	F=M	F=M, but depends on subpopulation
Ketoacidosis	±	+	+
Duration of insulitis	Long	Short	Short to long
Islet (auto)antibodies	±	±	+
Lymphopenia	-	+	-
Associated autoimmunity	Thyroiditis Parathyroiditis Hemolytic anemia Adrenalitis Intestinal nephritis Sialoadenitis Dacryoadenitis Colitis Myositis Neuritis Meningitis Osteomyelitis	Thyroiditis Gastritis	Hashimoto's thyroiditis Graves' disease Adrenalitis Addison's disease Gastritis Rheumatoid arthritis Vitiligo Pernicious anemia Polyendocrine syndroms Primary myxedema

Despite many years of extensive research, the pathophysiology underlying type 1 diabetes has remained largely obscure. The main reason for this is that clinical symptoms of diabetes do not develop until more than 80-90% of the β cell mass is destroyed. Since patients therefore do not present before the autoimmune attack is virtually finished, the steps that lead to the autoimmune attack remain unclear.

In order to gain more insight into the pathogenic mechanisms leading to β cell destruction, animal models have been developed. Amongst these are the nonobese diabetic (NOD) mouse and the BioBreeding (BB) rat. Both NOD mice and BB rats spontaneously develop a disease resembling human type 1 diabetes. These models provide us with the opportunity to study early phases in the process leading to diabetes. Moreover, the possibility of modifying their genetic background facilitates the study of genes that are potentially involved in diabetes development. Also other experiments aimed at the manipulation of the onset of diabetes would be ethically and technically impossible in humans, but can be performed in experimental animals.

There is, however, an important difference between human type 1 diabetes and its animal models. In contrast to NOD mice and BB rats, human type 1 diabetes patients differ significantly in genetic background as well as environment. Therefore, it is highly unlikely that processes leading to type 1 diabetes are comparable among patients. The animal models can thus be envisaged as case reports, and will probably not be representative of all human patients. Therefore, a prerequisite for the correct interpretation of observations from animal models in relation to human disease is a good knowledge of the aspects in which the models do or do not resemble the human disease process.

To enable a proper comparison of the animal models with human type 1 diabetes, this review will focus on the actual processes that take place at the cellular level in the pancreas in human type 1 diabetes and its animal counterparts. More specific, we will discuss the cells and cell products and their most likely roles in the pathophysiological process leading to diabetes. Given the vast literature on human type 1 diabetes and the animal models resembling the disease, the genetics of the disease and specific immune defects leading to autoimmunity are beyond the scope of this review. Other animal models for type 1 diabetes will also not be discussed here.

NOD mouse

Autoimmune diabetes in NOD mice

The NOD mouse was bred at the Shionogi Research Laboratories in Aburahi, Japan from a female mouse displaying severe glycosuria, which belonged to the CTS-subline of outbred ICR mice. An inbred strain was derived by brother-sister matings (19). Early in the program of inbreeding, an MHC-incompatible control line was developed from the CTS-subline of outbred ICR mice, now designated the nonobese nondiabetic (NON) mouse (20).

A nonobese diabetes-resistant (NOR) strain was also developed. This NOD-related MHC-syngeneic recombinant strain possesses ~12% C57/KsJ (BKs)-derived genes and therefore has a unique combination of resistance and susceptibility genes (21-23). NOR mice are resistant to both spontaneous and cyclophosphamide-induced diabetes (see below) and display only a mild inflammatory infiltrate surrounding the islets, so-called peri-insulitis, up to 9 months of age (21-24). Furthermore, the NOD mouse has been backcrossed to the C.B.-17*scid* mouse, resulting in the NOD*scid* mouse. This mouse lacks functional T and B lymphocytes and consequently develops neither lymphocyte insulitis nor diabetes (25).

Currently, several colonies of NOD mice are maintained throughout the world (26). Although they are supposed to be genetically identical, a direct comparison of diabetes incidence in two substrains maintained in a common environment indicated that substrain divergence is occurring (20). This is not surprising, given the likelihood that inbred NOD colonies worldwide are accumulating independent genetic mutations that will eventually lead to substrain divergence. Substrain and environmental differences lead to significant variation with respect to incidence of diabetes, time of onset and ratio of female to male diabetic mice. In the original colony, and in most derived colonies, the females are more prone to diabetes development than the males, and especially the incidence in male mice is variable (20). The differences between colonies indicate the importance of environmental factors in the development of diabetes, such as gnotobiotic environment, temperature and diet (18,26,27).

The first pathological symptom in NOD mice is the accumulation of leukocytes around the islets. This so-called peri-insulitis may be evident as early as 2-3 weeks of age (28,29), preceding diabetes symptoms by many weeks. The autoimmune reaction is directed against several autoantigens (4,6,24). Usually, NOD females become diabetic at about 19 weeks of age, while males become diabetic at about 24 weeks of age (26). By 30 weeks, typically 80% of female and 20% of male mice have become diabetic (18,27). Cyclophosphamide treatment can be used to compress and synchronize diabetes development in NOD mice. Administration of cyclophosphamide to NOD mice results in predictable acceleration of β.cell destruction leading to diabetes within 7 to 14 days and hyperglycemia occurring in 60% of female NOD mice (30,31). Onset of both spontaneous and induced diabetes is marked by the appearance of moderate glycosuria and by a non-fasting plasma glucose higher than 250 mg/dl. Both glycosuria and hyperglycemia become progressively more severe over a 3-4 week period during which weight loss and polyuria also occur. In addition to becoming severely hyperglycemic, diabetic mice are ketonemic, but, in contrast to human diabetes patients and BB rats, not ketoacidotic (32). Diabetic animals can survive for up to a month without exogenous insulin, but eventually insulin is required for survival (18,20).

The main genetic component determining the susceptibility for diabetes in NOD mice is the MHC, but other genes involved in immune function also play a significant role (33). Indeed, in addition to insulitis and diabetes, NOD mice develop several other local inflammatory processes (20,26) (Table 1). Furthermore, they show an increased incidence of a wide spectrum of neoplasms, in particular lymphomas, but also other tumors (20,26). The main clinical features of the NOD mouse model are summarized in Table 1.

APC initiate the islet attack

In NOD mice, there are several indications that insulitis is initiated by professional antigen-presenting cells (APC), including B cells (Table 2).These APC infiltrate the islets of Langerhans, independent of coincident T cell migration. In the islets, they pick up islet antigens, which they present in the pancreas-draining lymph nodes to T cells. Most likely, APC are needed for the generation of β cell-specific effector T cells.

Table 2: Evidence that APC initiate the islet attack in NOD mice and BB rats.

NOD mouse	BB rat
• Spontaneous insulitis in young NOD is primarily mediated by an influx of DC and Mϕ that precedesT cell accumulation in islets (34,35). • In both NODscid and NOR mice, there is an accumulation of DC and Mϕ around the islets without any accumulation of T cells (36,37). • Peri-insulitis is absent in NOD mice depleted of phagocytic cells (including Mϕ and DC precursors) by means of silica treatment early in diabetes development, even after acceleration with cyclophosphamide (38). However, silica has no effect after onset of invasive insulitis (38-40). • The blockade of Mϕ adhesion-promoting type-3 complement receptor (CR3 or CD11b/CD18) in vivo before adoptive transfer not only prevents intra-islet infiltration by Mϕ, but also by T cells and inhibits diabetes development, although the monoclonal antibody 5C6 that is used does not bind to T cells (41). • Peri-insulitis is absent in young NOD mice deficient in B cells, a defect that can be restored by transfer of B cells but not by immunoglobulin infusions (42).	• Myeloid cell influx precedes the infiltration with lymphocytes during spontaneous insulitis (43-48). • Treatment of DP-BB rats with silica, that almost completely prevents disease manifestation (49), suppresses both Mϕ and lymphocyte infiltration (48,50). • ConA-activated splenocytes from long-term silica treated DP-BB rats do not induce insulitis or diabetes in DP-BB neonates; in contrast to those from untreated DP-BB rats, probably as a result of a decrease in the number of Mϕ-dependent T lymphocytes and a reduction in NK cell cytotoxicity (51).

The cause of early APC infiltration is unknown. It has been hypothesized, based on human and BB rat studies, that virus-induced interferon-α (IFN-α) expression may be involved (52-54). Retroviruses have been suggested as possible candidate viruses for the triggering of β cell specific autoimmunity in NOD mice. Indeed, retrovirus particles, retroviral DNA and retroviral proteins have been detected in pancreatic islets of NOD mice (28,55-58). NOD β cells, in contrast to NON β cells, express not only type A, but also type C particles (57).

The pathophysiological relevance of the presence of these retroviruses is, however, unclear. Theoretically, virus-induced IFN-α expression can function as the trigger for APC

infiltration, but the evidence argues against this scenario. First, IFN-α expression in NOD islets could not be detected (53). Second, it has been shown that administration of polyionisinic-polycytidilic acid (poly I/C), a synthetic double-stranded polyribonucleotide that elicits inflammatory responses analogous to those observed during viral infection and that induces IFN-α expression in islets, prevents the development of diabetes in NOD mice (59). Thus, it seems unlikely that virus-induced IFN-α expression by NOD β cells is involved in the attraction of early infiltrating APC. Alternatively, the presence of a virus could possibly induce autoimmunity due to the presence of molecular mimicry between retroviral proteins and β cell autoantigens (60).

Several studies have investigated the type and location of early infiltrating APC in more detail. Mϕ and DC, potentially functioning as APC and involved in early peri-insulitis, are either constitutively present in the pancreas and subsequently home to the islets, or are recruited from the circulation. In the normal pancreas, APC expressing one or more of the markers CD11b (61-63), F4/80 (35,61-63), SER-4 (61), ER-MP23 (35), MOMA-1 (35) and CD13 (ER-BMDM1) (35) are present. They are usually found in different locations: lining the exocrine pancreatic acini, in connective tissues, in the perivascular area and around normal islets (35,61,62). Only $CD13^+$ APC are present, at low frequencies, within normal islets (35). From 3-4 weeks onwards, increased numbers of APC are found in NOD pancreas, mainly surrounding the islets and scattered in the exocrine region and in perivascular sites (28,35,62). These APC include the pancreas-derived cells expressing one or more of the markers F4/80 (35,62,63), CD11b (35,62,63), ER-MP23 (35) and MOMA-1 (35). Moreover, low numbers of newly attracted $Mac\text{-}2^+$ (35) and $ER\text{-}HR3^+$ (35) cells and high numbers of $CD11c^+$ (N418) DC (36), $NLDC\text{-}145^+$ DC (64) and $BM8^+$ Mϕ (35) start accumulating around the islets.

Early infiltrating APC have been shown to produce tumor necrosis factor-α (TNF-α) in NOD and NOD*scid* mice (36). It has been hypothesized that an early and local production of TNF-α in the islets acts to enhance the islets antigenicity and the subsequent activation of disease-causing lymphocytes (65-67). Moreover, since TNF-α in combination with other cytokines may be cytotoxic to β cells (see below), early expression of this cytokine may induce partial β cell dysfunction or destruction, facilitating uptake of β cell antigens by APC. In this respect, treatment of newborn NOD mice with neutralizing anti-TNF antibodies inhibits the development of both insulitis and diabetes (65).

As pointed out before, early infiltrating APC are thought to leave the pancreas and present antigen to lymphocytes in the pancreas-draining lymph nodes. These subsequently activated lymphocytes will then migrate to the pancreas.

Leukocyte traffic into the pancreas: highways of adhesion molecules

Infiltration or migration of lymphocytes into tissues generally occurs from postcapillary venules (68,69). Indeed, several authors report the presence of lymphocytes within pan-

creatic postcapillary venules of NOD mice (28,70-73). These venules show significant morphological alterations, which include dilation and activation of endothelium with local transformation to hypertrophic cells (35,74). This High Endothelial Venule (HEV)-formation is probably the consequence and not the cause of the lymphocyte infiltration, since the morphological changes are usually present near infiltrating lymphocytes (72) and can also be found after T cell transfer in newborn NOD mice (75). The presence of mononuclear leukocytes within the lumen of postcapillary venules is observed only as long as insulitis is present and may be explained as a recruitment of cells of the immune system from the blood (71).

All infiltrating mononuclear cells utilize vascular addressins and other adhesion molecules on these postcapillary venules to enter the pancreas. The upregulation of all these vascular adhesion molecules is limited to vessels that are adjacent to or surrounded by inflammatory cells (76). Thus, vascular adhesion molecule upregulation is also caused by local stimulation and cytokine release by the infiltrating leukocytes. The adhesion molecule pairs and their most likely role in diabetes development are summarized in Table 3.

Table 3: Involvement of pairs of adhesion molecules in diabetes development in NOD mice and humans. To the best of our knowledge, there are no data on the BB rat.

Adhesion pair	Function	NOD mouse	Human
MadCAM-1/LPAM-1	Leukocyte extravasation	+ (76-81)	?
PNAd/L-selectin	eukocyte extravasation ymphocyte trafficking in LN	+ + (79,81-83)	- ? (84)
ICAM-1/CR3	Mϕ extravasation	+	?
ICAM-1/LFA-1	Leukocyte extravasation Antigen presentation cell destruction	+ + -? (41,61,72,76,78,85-92)	+ - - (93-97)
VCAM-1/VLA-4	Leukocyte extravasation Entry CD4+ T cells into islets Lymphocyte trafficking in LN	+ + + (64,78-82,86,92,98,99)	- - ? (93,94)
LFA-3/CD2	Antigen presentation	?	- (93,95,96)

The first pair of adhesion molecules that is upregulated from 4 to 5 weeks onwards on endothelium and leukocytes in the NOD pancreas is MAdCAM-1/$\alpha 4\beta 7$(LPAM-1) (76,78,79,81). Another adhesion pair that is upregulated already in early stages of insulitis is PNAd/L-selectin (also designated MEL-14, LECAM-1, LAM-1 and CD62L) (76,78,86).

Both MAdCAM-1/LPAM-1 and PNAd/L-selectin are involved in early leukocyte extravasation.

In later stages of insulitis, expression of ICAM-1, which is normally absent on islet endothelial cells (72,76,78,85,86), is increased (86,87). ICAM-1 probably has several roles in the development of insulitis, partly dependent on the ligand that it binds. The ICAM-1/CR3 (also designated Mac-1 and CD11b/CD18) interaction is involved in intra-islet infiltration by monocytes and monocyte-derived cells (41). A second ICAM-1-ligand that is important in the insulitis process is LFA-1, that is expressed on virtually all islet-infiltrating cells (76,78,85,87). Unstimulated T cells are unable to use LFA-1 for binding to endothelial cells (88), but activation of T cells by various stimuli has been demonstrated to result in an enhanced utilization of LFA-1 (100). Besides a role in leukocyte extravasation, the ICAM-1/LFA-1 pathway is probably involved in other processes during insulitis. The observed ICAM-1 hyperexpression on the T cell surface can enhance antigen presentation by APC to T cells by facilitating the adhesion. Moreover, interactions between two T cells can also be mediated by bi-directional LFA-1 and ICAM-1 interactions (61,76,87). Finally, a role for the ICAM-1/LFA-1 pathway in β cell destruction has been suggested, but this is controversial. Freshly isolated pancreatic endocrine cells from young NOD mice without insulitis do not express ICAM-1, but induction of ICAM-1 by inflammatory cytokines (IFN-γ, TNF-α, IL-1β) *in vitro* has been reported and may theoretically occur in the inflamed islets as well (85,91). Cytotoxic T cells may use LFA-1 to interact with inflammation-induced ICAM-1 on β cells, which facilitates target cell lysis. In favor of this hypothesis, ICAM-1 expression on NOD-derived insulinoma cells enhances the susceptibility of these cells to CTL-mediated lysis (85). Moreover, if the ICAM-1/LFA-1 interaction is blocked only during late and not during early stages of insulitis, there is partial protection from the development of spontaneous diabetes (85,87). The controversy concerning the role of LFA-1/ICAM-1 in β cell destruction is fueled by the fact that blocking the ICAM-1/LFA-1 interaction fails to prevent diabetes induced by cyclophosphamide and induced by transfer of diabetogenic splenocytes or islet-derived mononuclear cells (87,92). Moreover, several investigators have not been able to detect ICAM-1 *in situ* on diabetic NOD islet cells (61,76,78,87). Even after cyclophosphamide-accelerated insulitis, which is characterized by a massive local IFN-γ production, *in situ* ICAM-1 expression was not found (86). In contrast, one study using immunoelectron microscopy has shown ICAM-1^+ islet cells adjacent to lymphocytes. Such localization of ICAM-1 would be difficult to detect by light microscopy, since the expression was limited to cells facing infiltrating lymphocytes that strongly expressed ICAM-1 on their surface (85). In conclusion, these studies indicate that the ICAM-1/LFA-1 interaction may be involved, but is not essential in β cell destruction in late stages.

Another adhesion pair that is upregulated when the pancreas is already infiltrated, is VCAM-1/VLA-4 (64,81,86,92). VCAM-1 is induced in islets with moderate infiltrates (64,81,86,92). VLA-4 is constitutively expressed on leukocytes, and the VCAM-1/VLA-4 interaction may therefore be implicated in the entry of cells into the pancreas (78,82). However, as for LFA-1, VLA-mediated adhesion depends upon the activation status of the

individual cells (99). Remarkably, after blocking the VCAM-1/VLA-4 interaction, $CD4^+$ T cells remained at the outer edges of the islet, while $CD8^+$ T cells penetrated into the islet (92). Thus, the VCAM-1/VLA-4 pathway may play a role in entry of cells into the pancreas and also in allowing entry of $CD4^+$ T into islets.

The lymphoid actors: major roles for T and B, but not NK cells

The lymphoid cells in insulitis consist of B and T lymphocytes and NK cells. In NOD mice, B cells appear to be essential for antigen presentation and T cells for insulitis expansion. NK cells are probably not crucially involved in NOD diabetes.

Several researchers have observed B lymphocytes infiltrating the NOD pancreas (63,78,101,102). Usually, they are not detected at the early stages of infiltration, but increase in number with the progression of the lesion (29,74,75,78). During the later stages of spontaneous insulitis and during insulitis recurrence in islet grafts, B lymphocytes represent about 30% of infiltrating cells (78,101,102). In addition to B lymphocytes, a few plasma cells were observed in some studies (28,29,74). Both B lymphocytes and plasma cells are reported to be situated in the periphery of infiltrating cell masses (28,29,103). Occasionally, B lymphocytes form a lymphoid follicle-like structure (74,104). In cyclophosphamide-accelerated diabetes, B cells are virtually absent (105).

B cells play an essential role in the pathogenesis of autoimmune diabetes. NOD mice made deficient for B lymphocytes, either genetically (42,106,107) or by treatment with specific antibody (108,109), are resistant to diabetes development. Theoretically, B cells can be involved in two stages of diabetes development. If they develop into plasma cells, they can contribute to diabetes pathogenesis via their ability to secrete auto-antibodies that bind pancreatic β cells. Alternatively, B cells may be involved as APC in the stimulation of T cells. Several studies suggest that the role of B cells in the presentation of autoantigens is essential in diabetes pathogenesis, whereas the production of auto-antibodies is merely an epiphenomenon. In B cell-deficient NOD mice, diabetes susceptibility is restored if these mice are reconstituted with B lymphocytes, but not by infusions of immunoglobulins from diabetic NOD donors (42). Moreover, B cell-deficient mice have no spontaneous T cell response to certain β cell autoantigens, while responses to control antigens are intact (42,110). This indicates that B cells are essential for the development of T cell reactivity against specific β cell antigens. The specificity for autoantigens may be the result of the unique capability of B cells to take up antigens selectively (42,110). Finally, NOD mice with a B cell-restricted $I\text{-}A^{g7}$ deficiency (NOD B^{CIID}), and thus with B cells incapable of antigen presentation, are resistant to diabetes development, although rare foci of peri-insulitis are present in old animals (111). Therefore, B cells appear to be essential APC for the initiation of certain β cell autoreactive T cell responses. Purified T cells from NOD donors can efficiently transfer disease to either B cell-depleted neonatal recipients or NOD*scid* recipients (112,113). Thus, once dia-

betogenic T lymphocytes have been generated, they can clearly mediate destruction of pancreatic β cells in the absence of B cells.

The production of auto-antibodies by B cells thus appears not to be essential for diabetes pathogenesis. This does not mean that autoantibodies are not involved at all, as they theoretically can contribute to β cell damage. Auto-antibodies to islet cells can already be found in serum of NOD females from 2 to 3 weeks of age. It is, however, unclear whether these IgG antibodies are endogenous or of maternal origin (114,115). Natural binding of these auto-antibodies to the β cell surface has been detected at the same age (114,116), although one study did not find immunoglobulin deposition in the pancreas (29). There is, however, no evidence that these auto-antibodies are pathogenic. Complement-mediated islet cell lysis does not occur, since NOD and NOD*scid* mice, as approximately 30% of mouse strains, lack complement activity due to a deficiency in C5, and therefore can not form the C5b-9 membrane attack complex (117-119).

B cells are thus essential for the development of diabetes in NOD mice, most likely in their capacity as APC. Several observations indicate that T cells are also crucial in the process leading to diabetes in NOD mice. Diabetes can be transferred by splenic T cells from diabetic (75,113,120-125) or prediabetic (113) mice and by particular T cell clones (126-131). In reverse, diabetes does not develop in mice made T cell deficient, either genetically (25,132) or by experimental treatments (133-135). In addition, cyclosporin (136) and FK-506 (137), which amongst others suppress cytokine production by $CD4^+$ T lymphocytes, suppress diabetes development.

Indeed, most studies report T cells to be the dominant cell population in infiltrates of NOD mice with spontaneous insulitis (29,63,78,103), in NOD or NOD*scid* mice after adoptive transfer (75,105,138), and in NOD mice after diabetes acceleration by cyclophosphamide (105,139).

What is the phenotype of the T cells present in the infiltrates? In virtually all studies, higher numbers of $CD4^+$ cells than $CD8^+$ cells are found in the pancreas, both in spontaneous disease (29,87,103) and after transfer (75,140). However, in spleen and peripheral lymph nodes, $CD4^+$ cells are also more prominent than $CD8^+$ cells. The one study that has compared the CD4/CD8 ratio in the pancreas with that in the spleen has found that the ratio in pancreases with spontaneous disease is higher than the ratio in spleen, suggesting selective accumulation of $CD4^+$ T cells in the lesions (78). During the development of spontaneous insulitis, however, a progressive decrease in the proportion of $CD4^+$ cells and consequently an increase in the fraction of $CD8^+$ cells took place (63,78). In T cell or splenocyte transfer studies and in islet transplantation studies, a similar or even lower CD4/CD8 ratio is found in pancreas compared to spleen (75,101,138). This again suggests a role for $CD8^+$ T cells in β cell destruction, since both adoptively transferred diabetes and diabetes recurrence in islet grafts reflect the late stages of the disease. Taken together, these in situ studies thus suggest a role for $CD4^+$ cells in early phases of insulitis, and a role for $CD8^+$ cells in islet destruction.

Early transfer studies provided evidence that both $CD4^+$ and $CD8^+$ T cells are required to provoke or accelerate disease. This holds true when either splenocytes from diabetic or prediabetic NOD donors or autoreactive NOD islet-derived $CD4^+$ and $CD8^+$ T cell clones are transferred into irradiated young NOD or NOD*scid* recipients (113,122-125,141). $CD4^+$ T cells, alone, can invade the islets, but, in general, diabetes develops only when $CD8^+$ T cells are also present. $CD8^+$ T cells, alone, do not infiltrate the islets (113,124,125,142) and therefore appear to require signals from $CD4^+$ T cells to effect β cell damage. This hypothesis is supported by the finding that $CD4^+$ T cells precede $CD8^+$ T cells in entering into the islets when whole splenocytes from diabetic donors are transferred into healthy recipients (61,75). Hence, $CD4^+$ T cells seem to facilitate the initial entry of $CD8^+$ T cells into the islets. The picture therefore emerges that both CD4 and $CD8^+$ T cells are essential: $CD4^+$ T cells especially in the initiation phase and $CD8^+$ T cells in β cell destruction. Supportive evidence for this view is presented in Table 4.

Table 4: Evidence that, in NOD mice, $CD4^+$ T cells initiate insulitis, whereas $CD8^+$ T cells are involved in β cell destruction.

- $CD8^+$ T cell lines and clones have been isolated with in vitro and in vivo cytotoxic activity against β cells (126,131,141,143-146).
- Although transfer of the β cell reactive $CD4^+$ T cell clone BDC2.5 by itself is able to induce diabetes in young NOD mice, cotransfer of CD8-enriched T cells was required to induce disease in NOD*scid* mice (128)
- The need for $CD4^+$ T cell help in diabetes transfer experiments can be overcome by high numbers of in vitro activated cloned autoreactive $CD8^+$ T cells (126,142).
- Mononuclear cells dissociated from NOD islets and cultured in the presence of IL-2 are composed mainly of H-$2K^d$-restricted $CD8^+$ T cells exhibiting a lytic activity to islets (144). Depletion of $CD4^+$ T cells from these islet-derived cells did not influence islet cell lysis in vitro, whereas depletion of $CD8^+$ T cells almost completely abolished it (144).
- Electron microscopic findings indicate that $CD8^+$ T cells directly attack the pancreatic islets of NOD mice (147).
- Diabetes, but not insulitis incidence, is reduced when adult NOD mice are injected with antibodies against MHC class I or CD8 (148). Conversely, both insulitis and diabetes are prevented when adult NOD mice are treated with anti-CD4 (149).
- NOD mice lacking the CIITA (class II transactivator) molecule are deficient in MHC class II expression and peripheral $CD4^+$ T cells. $CD8^+$ T cells from these mice are only able to transfer insulitis and diabetes to NOD*scid* recipients if NOD $CD4^+$ T cells are present (142).

$CD8^+$ T cells probably play an additional role in the autoimmune response. NOD mice carrying a null mutation at the β2-microglobulin (β2-μ) locus, and thereby lacking MHC class I molecules and $CD8^+$ T cells, are not only diabetes- but also insulitis-resistant (150-153). This may indicate that $CD8^+$ T cells also play a role in the initiation of insulitis. Moreover, the development of insulitis is severely inhibited when NOD mice are treated with an anti-CD8 monoclonal antibody during a discrete age window from about 2 to 5 weeks after birth (154). Transfer studies indicate that $CD4^+$ T cells from these protected mice,

although they were isolated after repopulation of the CD8 compartment, are not capable of transferring insulitis (154). This indicates that $CD4^+$ T cells somehow need $CD8^+$ T cells during a discrete age window to be capable of initiating insulitis.

A more precise definition of the role of $CD8^+$ T cells comes from the observation that in certain situations, the absolute need for $CD8^+$ T cells in diabetes development can be overcome. For example, when large numbers of cloned $CD4^+$ T cells are transferred, not all of these clones need $CD8^+$ T cells to provoke disease (127,130,155,156). This apparent lack of $CD8^+$ T cell requirement may be explained by recruitment of host $CD8^+$ T cells. However, similar observations have been made in transfer studies in which the host was deficient in $CD8^+$ T cells. When only $CD4^+$ T cells isolated from diabetic NOD mice are transferred into NOD*scid* recipients, both insulitis and diabetes develop, while this is not the case when prediabetic splenocytes are used (113). Similarly, certain islet-reactive $CD4^+$ T cell clones isolated from diabetic NOD mice are able to transfer diabetes to NOD*scid* recipients (128,129). Moreover, the complete depletion of $CD8^+$ T cells does not affect insulitis development in a T cell receptor transgenic mouse strain with a $CD4^+$ T cell repertoire highly skewed for an anti-islet cell reactivity (157). Finally, destruction of syngeneic islet grafts by spontaneously diabetic NOD mice can be prevented by depletion of $CD4^+$, but not $CD8^+$ cells of the host (158). These studies suggest that the need for $CD8^+$ T cells in disease initiation can be overcome when large numbers of autoreactive $CD4^+$ T cells and/or highly activated $CD4^+$ T cells are present. This suggests that $CD8^+$ T cells play a role in the generation of autoreactive $CD4^+$ T cells, possibly by initiating β cell necrosis (113). This early β cell death apparently leads to the release of autoantigens, resulting in the priming and expansion of autoreactive $CD4^+$ T cells.

In contrast to what is observed for T cells, no or only few NK cells are found located close to islet cells and scattered among infiltrating cells in NOD mice (29,74,138). One *in vitro* study has noted a small number of asialo-$GM1^+$ cells, presumably NK cells, in islet-derived leukocytes, which are able to lyse NK-sensitive targets (144). However, anti-asialo-GM1 only slightly reduced the cytotoxicity of islet-derived cells to NOD islet cells, arguing against a major role for NK cells in β cell cytotoxicity. Moreover, in this *in vitro* study, the immune attack against NOD islet cells has been demonstrated to be MHC-restricted (144). It therefore seems unlikely that NK cells play a significant role as cytotoxic effector cells in islet destruction.

To summarize the role of lymphoid cells, both B and $CD8^+$ T cells seem to be crucially involved in the initiation of insulitis, the former as APC and the latter as inducers of early antigen release. $CD4^+$ T cells come into play thereafter, and activate $CD8^+$ T cells for final β cell cytotoxicity. There is no evidence that autoantibodies or NK cells are important in β cell destruction.

The Th1-Th2 paradigm in insulitis expansion

The type of cytokines that is produced in an insulitis process determines the final outcome of the β cell-directed autoimmune reaction. In general, if Th1 cytokines such as IFN-γ are produced by islet-infiltrating mononuclear cells, β cell destruction occurs and diabetes will develop. If, on the other hand, Th2 or Th3 cytokines such as IL-4, IL-10 and TGF-β are produced, there is protection from β cell destruction and diabetes. Evidence for this notion comes from both histological and functional studies.

Early peri-insulitis in NOD mice is characterized by a dominance of cells producing Th2 cytokines (159-162). However, later stages of insulitis are associated with a clear shift towards Th1 cytokine profiles (163-167). The acceleration of diabetes development induced by treatment of NOD mice with cyclophosphamide also correlates with an increased expression of IFN-γ (139,161,168,169), while Th2 cytokines are still present (160,161). In addition, diabetes development in different mouse strains can be correlated with cytokine profiles in the islets. Low incidence NOD mice, such as mice originating from low incidence colonies, NOR mice and male NOD mice, have elevated levels of Th2 and/or decreased levels of Th1 cytokines in islet-infiltrating mononuclear cells compared to high incidence NOD mice (34,37,139,164,165,170). In conclusion, these data indicate that the progression to invasive insulitis and β cell destruction requires Th1 cells in islet lesions.

In addition to these descriptive studies, functional studies have underlined a role for Th1 in β cell destruction and Th2 in diabetes protection. β cell-specific Th1 clones efficiently transfer disease to syngeneic young NOD recipients, whereas Th2 clones do not (157,171). In NOD*scid* mice, Th2 clones have been able to cause generalized pancreatitis, islet cell necrosis and diabetes, but the histopathological process in these animals is completely different from the spontaneous NOD insulitis (172). In addition, neutralizing Th1 cytokines byinjection of specific antibodies, or by counterregulatory Th2 cytokines, prevents β cell destruction (139,168,173-180). Moreover, treatments known to prevent diabetes in NOD mice are associated with decreases in Th1 and/or increases in Th2 cytokine expression (162,164,165,181-189).

Interestingly, the skewed Th1/Th2 balance found in islet-associated mononuclear cells is not reflected in the spleen, as has been shown both in islets during spontaneous insulitis (164) and in islet grafts with recurrent diabetes (101). This suggests that the Th1/Th2 balance produced by islet-infiltrating cells may be regulated locally in the islet. Alternatively, the recruitment of Th1 and Th2 cells may be different. In this regard, there is a striking difference in the spatial distribution of IFN-γ^+ and IL-4^+ cells even within the infiltrate: IL-4^+ cells occur throughout the infiltrated area, while IFN-γ^+ cells are restricted to areas adjacent to β cells (161). Apparently, Th1, but not Th2, cells demonstrate an attraction towards β cells, probably related to a unique dependence of Th1 activation on islet antigen specificities or quantities present on or near β cells.

Thus, the conversion from a Th2- to a Th1-type-infiltrate is essential for the transition from peri-insulitis to destructive insulitis and thus for diabetes development. There are two major candidate factors that can promote this transition: IL-12 and IL-18 (also designated IFN-γ inducing factor or IGIF). Both cytokines are exclusively produced in Mϕ and other APC, and both have been shown to drive Th1 responses. Several studies indicate that IL-12 and IL-18 are indeed involved in the activation of Th1 cells in NOD mice. The upregulation of IL-12 and IL-18 expression in the insulitis lesion correlates with the development of spontaneous diabetes, cyclophosphamide-accelerated diabetes and recurrent diabetes in islet grafts in NOD mice, possibly via the induction of Th1 cytokines (161,169,188,190). Moreover, the daily treatment of prediabetic NOD mice with large doses of IL-12 results in a rapid progression towards insulitis and diabetes, coinciding with an increased Th1 cytokine production by islet infiltrating cells (191). In contrast, a low dose of IL-12 or IL-18 suppresses both spontaneous and cyclophosphamide-accelerated diabetes development, probably because such a dose is too low to effectuate a Th1 response, but high enough to trigger counterregulation by the immune system, limiting Th1 reactivity (163,192,193). The administration of an IL-12 antagonist results in an increase of Th2 activity and is associated with a reduction in spontaneous and cyclophosphamide-accelerated diabetes (194). Moreover, the cyclophosphamide-accelerated diabetes development is suppressed in NOD mice with a disrupted IL-12 gene (195). A fraction of animals still developed diabetes, possibly due to IL-18-mediated Th1 induction and subsequent β cell destruction and disease development (190).

In summary, the progression from benign to destructive insulitis appears to occur via the release of Th1-activating cytokines such as IL-12 and IL-18. Since these cytokines are exclusively produced by Mϕ and other APC, these data suggest that APC within islets drive the progression from a non-destructive insulitis towards diabetes. Further evidence for the crucial role of APC comes from the observation that T cells in a Mϕ-depleted environment lose their ability to differentiate into β cell-cytotoxic T cells. This T cell differentiation block is associated with a decreased Th1/Th2 ratio and can be abrogated by IL-12 (196).

The hypothesis of aberrant MHC expression revisited

The expression of MHC-peptide complexes on islet cells is essential for the susceptibility of these cells to autoimmune attack. In NOD mice, both an islet hyperexpression of MHC class I molecules and an aberrant islet expression of MHC class II molecules have been suggested to be involved in diabetes pathogenesis.

In normal pancreas and in the pancreas of young NOD mice, MHC class I is only weakly expressed, mainly on the vascular endothelium and on constitutively present Mϕ (61). However, MHC class I hyperexpression is induced on endocrine islet cells and adjacent exocrine tissue during the development of spontaneous (61,63) and cyclophosphamide-accelerated insulitis (56). This hyperexpression is especially associated with infiltrates (56,61), and the infiltrating cells themselves also exhibit strong MHC class I labeling (56,61).

The association between islet MHC class I hyperexpression and the presence of infiltrates suggests that the hyperexpression is induced by cytokines produced by the infiltrate. Several studies suggest IFN-γ to be involved. IFN-γ has been shown to induce MHC class I expression on islet cells *in vitro* (197). Moreover, anti-IFN-γ, which is known to decrease the degree of insulitis and to prevent hyperglycemia, markedly reduces expression of MHC class I on islet cells (56). Finally, MHC class I upregulation is not found in NOD mice with β cells that are unresponsive to IFN-γ due to dominant negative mutant IFN-γ receptors, despite the presence of insulitis (198). Together, these findings clearly indicate the *in vivo* relevance of IFN-γ for MHC class I induction.

Another factor that can theoretically induce MHC class I hyperexpression is infection with viruses (199). We already discussed the observation of the presence of retrovirus in NOD islets. However, retrovirus expression is unlikely to be the direct stimulus for MHC class I overexpression as this overexpression is delayed for 1 week after cyclophosphamide treatment, whereas type C retrovirus has been amplified by PCR from NOD mouse islets immediately after administration of cyclophosphamide (56,139). Moreover, retrovirus expression in islets would not explain why exocrine cells, not observed to express retrovirus particles, also overexpress MHC class I (56).

In conclusion, islet MHC class I hyperexpression is induced by cytokines produced by the infiltrating cells. Although MHC class I overexpression may not be required for initiation of insulitis, it may play a role in the amplification and full development of the pathology. In this respect, it is likely that overexpression of MHC class I facilitates the targeting of MHC class I restricted cytotoxic T cells to islet autoantigens (63).

For MHC class II expression, an aberrant expression on islet cells has been suggested to contribute to diabetes development in NOD mice. In the normal pancreas, either from young NOD or from control mice, MHC class II is expressed by Mϕ or DC present in the exocrine pancreas and also sporadically present within the islet (200,201). Endocrine cells are MHC class II negative (61,200,201). During the development of insulitis, MHC class II expression is induced on ductal epithelial cells and vascular endothelium both during spontaneous insulitis (72) and after adoptive transfer of diabetogenic splenocytes or T cells (61,75). Moreover, MHC class II is also expressed by infiltrating cells (both APC and lymphocytes) in spontaneous insulitis, cyclophosphamide-accelerated insulitis and after adoptive transfer (29,56,61,72,73,102). The observation that vessel-associated MHC class II expression can be induced by the injection of effector cells, as is the case in adoptive transfer models, suggests that this expression is a consequence of the effector phase rather than an initial triggering event. In this sense, vessel-associated MHC class II expression seems to parallel the expression of vascular addressins, a phenomenon which also is induced by the infiltrating cells (see before).

The expression of MHC class II on endocrine cells is relevant, since it has been proposed that β cells may become APC after aberrant induction of MHC class II on their membrane (by, for instance, a viral infection). This is summarized in the so-called "aberrant MHC

class II hypothesis of endocrine autoimmunity" (202). However, most studies report that endocrine cells remain MHC class II negative during the development of insulitis. This is the case for endocrine cells during both spontaneous and cyclophosphamide-accelerated and during adoptively transferred insulitis (56,61,64,75,102,201). Only two studies show an induction of MHC class II on β cells in NOD females from early insulitis onwards (63,203). In one of those studies (203), the same expression was seen in control pancreases, thus raising questions about the specificity of the staining. The contradictory findings about MHC class II expression on endocrine cells are at least partly related to the use of different antibodies to detect it. Another cause may be the presence of MHC class II^+ leukocytes in islet cell populations. Islet cells in NOD mice express MHC class II at all time points when examined by FACS-analysis (204). However, subsequent double stainings showed that this MHC class II expression could be attributed to $CD45^+$ intra-islet leukocytes at all ages in the NOD mouse (205). In summary, expression of MHC class II is probably not induced on endocrine cells during diabetes development in NOD mice.

Even if MHC class II is expressed on endocrine cells, several observations suggest that the significance of β cells presenting antigens in NOD insulitis may be questioned. In NOD mice given IFN-γ together with TNF-α, a significant and uniform induction of MHC class I and MHC class II on ductal and acinar exocrine cells is found, as well as low level MHC class I on β cells (69). However, this endocrine MHC class II expression does not lead to an activation or enhancement of β cell autoimmunity, in contrast, it may actually suppress it. Moreover, induction of MHC class II on islet cells before transplantation by incubation with TNF-α did not induce a rapid rejection, but in contrast prolonged the survival of islet allografts (206). Indeed, several studies using transgene-directed expression of MHC antigens on β cells suggest that non-lymphoid cell expression of MHC class II is not stimulatory and may in fact be tolerogenic (207-209). Thus, the abnormal expression of MHC class I or MHC class II antigens by endocrine cells per se is insufficient to induce an autoimmune response. Moreover, it is not only insufficient but also not required, since insulitis and diabetes can be generated even when both the vascular endothelium and pancreatic β cells are genetically incapable of both MHC class I- and MHC class II-restricted autoantigen presentation (64).

In conclusion, MHC class II-restricted presentation of autoantigens by β cells does not seem to play a major pathogenic role in the triggering of NOD insulitis. In unmanipulated NOD mice, MHC class II is most likely not expressed by β cells at all, while artificially induced expression actually attenuates the autoimmune process.

$CD8^+$ T cells and Mϕ are major suspects for β cell destruction

Several cellular mechanisms have been proposed to operate in β cell killing. These include $CD8^+$ T cells that may effect β cell damage by the release of pore-forming molecules or by Fas-mediated apoptosis. Moreover, $CD4^+$ T cells in conjunction with Mϕ are also able

to cause β cell death. The soluble factors that play a role in this latter mechanism will be discussed in the next paragraph.

The first mechanism by which β cells can be destroyed is via direct interaction with cytotoxic $CD8^+$ T cells. As outlined earlier in this review, $CD8^+$ T cells indeed play an essential role in β cell destruction, although the mechanism is still incompletely understood. Cytotoxic CD8+ T cells may effect β cell damage by the release of granzyme and pore-forming molecules such as perforin (also designated cytolysin). Alternatively, the induction of Fas-mediated apoptosis can cause β cell death. Several studies indicate that pore-forming molecules may be more essential for diabetes development in NOD mice than Fas-mediated apoptosis.

$CD8^+$ cells expressing perforin are found in islets during spontaneous as well as transferred diabetes (138,159). In transfer recipient mice, perforin is mainly expressed in islet inflammatory cells before and during onset of glycosuria, which suggests a correlation with islet cell lysis (138). However, only a small percentage (<10%) of islet-infiltrating $CD8^+$ T cells acquired detectable levels of perforin antigen (138). Also the gene for granzyme A is expressed *in vivo* in islet-infiltrating cells during development of autoimmune diabetes in NOD mice (103). Similar to $CD8^+$ T cells expressing perforin, granzyme A $mRNA^+$ cells occur at a very low frequency, but the number of granzyme A $mRNA^+$ cells increases with the development of insulitis (103). It should be realized that even a low incidence of cells expressing pore-forming molecules over a longer period of time may be sufficient to mediate considerable tissue damage and, therefore, may be relevant to autoimmune insulitis in NOD mice (103). This is certainly true for perforin, since perforin-deficient NOD mice show a significantly delayed and reduced diabetes incidence (210).

Besides pore-forming molecules, $CD8^+$ T cells can use FasL to kill Fas^+ β cells. Whereas normal mouse islets do not express Fas, its expression can be induced by pro-inflammatory cytokines such as IL-1 and TNF-α (211-213). The upregulation of Fas by cytokines is independent of NO production (213), in contrast to what has been suggested for human islets (214). *In vivo*, Fas expression is increased in islets of NOD females at 15 weeks of age as compared to NOD males (215). Fas^+ β cells can be killed by $FasL^+$ T cells. Alternatively, islet cells may commit "suicide" due to cytokine-induced simultaneous expression of Fas and FasL. However, the latter possibility is excluded by the finding that NOD islet β cells are $FasL^-$, in contrast to earlier reports in which antisera with doubtful specificity for FasL have been used (213). On basis of these observations, Fas-mediated apoptosis may be a major mechanism of β cell destruction by $CD8^+$ T cells in NOD mice.

Indeed, results from some early studies suggest that Fas is essential for diabetes development in NOD mice. Fas^- NOD^{lpr} mice are resistant to spontaneous disease (212,216,217) and disease transferred by a $CD8^+$ T cell clone (212) or diabetogenic splenocytes (216,217). However, the absence of insulitis in these mice complicates the interpretation of these findings, since it can be expected that infiltrating cells home to the islet but are unable to kill β cells. One study has suggested that the absence of insulitis is related to

a block in the initiation of β cell autoimmunity (217). In such a scenario, transferred autoreactive T cells may initially damage β cells through the Fas-FasL system, and then subsequently destroy fragile target cells by other mediators such as perforin and cytokines. Arguing against the involvement of the Fas/FasL system in the initiation of insulitis is the finding that Fas staining on β cells can not be detected in young mice (213). However, it should be realized that Fas-expressing β cells may be short-lived because of their rapid destruction by $FasL^+$ T cells, and may therefore not be easily detectable. Alternatively, the diabetes resistance of Fas^- NOD^{lpr} mice could be explained by the substantially altered T and B cell immunity that characterizes these mice. The finding that NOD spleen cells are unable to survive in NOD^{lpr} mice further complicates the interpretation of the transfer studies (216). Therefore, instead of transferring diabetogenic NOD splenocytes into NOD^{lpr} mice, NOD^{lpr} islets have been transplanted into NOD mice to study diabetes recurrence as a model for the effector phase of β cell killing. Although NOD^{lpr} β cells can resist autoimmune attack somewhat better than NOD β cells, they can not reverse diabetes in spontaneously diabetic recipients and are eventually destroyed (216,218,219). Moreover, islet-specific cloned T cells destroy Fas^- islets as efficiently as Fas^+ islets (218). In addition, administration of anti-FasL antibody does not influence the development of either cyclophosphamide- or adoptive transfer-accelerated diabetes (219). Also, *in vitro*, anti-Fas antibody and FasL transfected cells do not exert cytotoxicity against β cell lines or pancreatic islets (220).

In conclusion, it is unlikely that Fas-mediated lysis is a major effector pathway in the final phases of β cell destruction. A role in the initiation phase of this destruction, however, can not be excluded. The generation of transgenic mice with FasL-insensitive β cells, but with a FasL sensitive immune system and thus a normal immune function, should ultimately provide conclusive evidence.

In contrast to direct cytotoxicity by $CD8^+$ T cells, $CD4^+$ T cells can mediate autoimmune β cell destruction in a manner analogous to a delayed type hypersensitivity (DTH) response that does not require cell-cell contact. As described earlier, in some cases $CD4^+$ T cells alone can transfer insulitis and diabetes and mediate graft rejection without the need for $CD8^+$ T cells. Since both MHC-matched and mismatched islet grafts in NOD mice have been shown to be destroyed by $CD4^+$ T cells, killing is not mediated via direct MHC class II-restricted $CD4^+$ T cell cytotoxicity (158). In addition, β cells do not express MHC class II (see before). $CD4^+$ T cell induced β cell damage may thus be mediated by factors such as IL-1, TNF-α and IFN-γ secreted by the T cells themselves and / or by locally activated Mϕ (158,221). Recently, it has been suggested that a similar interaction may occur between $CD8^+$ T cells and Mϕ (222). Both *in vitro* and *in vivo* studies suggest a role for Mϕ in final β cell killing. *In vitro*, activated Mϕ decrease glucose-induced insulin release (223). *In vivo*, the influx of $BM8^+$ Mϕ correlates with destructive insulitis (35,104). The selective destruction of β cells, with sparing of other islet cell types, may then be interpreted as a selective sensitivity of β cells to damage mediated by soluble factors, although this does not rule out the contribution of β cell-specific cytotoxic $CD8^+$ T cells. Indeed, blocking the effects of

Mϕ-derived TNF only partially protects NOD mice from diabetes; blocking perforin effects in addition, results in complete prevention (224).

Cytokines and free radicals are major β cell destructive weapons

Several cytokines have been related to β cell destruction in the NOD mouse. The most important of these are IL-1, TNF-α and IFN-γ. To study their role in diabetes development, the kinetics of their production have been related to β cell destruction.

IL-1β mRNA levels in isolated islet-infiltrating mononuclear cells progressively increase from 5 weeks of age to diabetes onset, indicating a possible role in β cell destruction (164). However, IL-1β mRNA levels in islet-infiltrating cells are identical for NOD females and males at all ages (159,164) and in islet grafts of complete Freund's adjuvant (CFA)-protected and control animals during diabetes recurrence (189). In contrast, IL-1α mRNA levels increase from 5 weeks of age to onset of diabetes, and are higher in islet-derived mononuclear leukocytes from diabetes-prone NOD females than in those from CFA-protected NOD females or NOD males (165). It should, however, be realized that an increase in mRNA levels does not automatically lead to an increase in biologically active protein, so these studies should be interpreted with caution.

Statistically significant differences are not found for TNF-α in islet-infiltrating cells in islet grafts from mice with recurrent diabetes versus recurrent prediabetes (101), for diabetes-prone NOD females and NOD males at all ages (159,164), and for CFA-protected and control animals during spontaneous diabetes (164) and islet graft rejection (189). Thus, TNF-α does not appear to mediate final β cell destruction. In contrast, as discussed before, TNF-mediated cytotoxicity is essential for the initiation of diabetes (65). NOD*scid* mice that are deficient in TNF receptor 1 (p55/TNF-R1) are protected from diabetes transfer by splenocytes from mice that are transgenic for a diabetogenic T cell receptor (218). In addition, the specific lack of TNF-R1 on islet cells altered the ability of cloned diabetogenic $CD4^+$ T cells to establish insulitis and subsequently destroy islet β cells (218). Thus, in this T cell receptor-transgenic NOD mouse model, an islet response to TNF-α is essential for the propagation of the $CD4^+$ autoreactive T cells and subsequent diabetes development. The nature of this response could be an increase in antigen delivery either in direct response to TNF-α or as a result of islet cell death (218).

Intra-islet TNF-α can be produced by infiltrating Mϕ as well as T lymphocytes. In early studies, T cells were shown to produce TNF-α mRNA in late stages of insulitis and β cell destruction (103,225,226). Furthermore, studies in NOR mice have shown that TNF-α mRNA correlates with CD3ε transcripts (37), linking TNF-α production to the presence of T cells. However, one study shows that TNF-α protein expression in situ in both early and late stages correlates very well with $CD11c^+$, $F4/80^+$ and Mac-1^+ APC labeling, and poor-

ly with $CD4^+$ and $CD8^+$ T labeling (36). Alternatively, based on studies using α and β cell lines, it has been suggested that β cells, but not α cells, may be induced by IL-1 to produce TNF-α, which would then be taken up by APC (227,228). Because TNF-α has been shown to potentiate β cell cytotoxicity of IL-1 and IFN-γ (229), TNF-α production *in situ* by β cells may be self-destructive. It is, however, unclear whether the responses of α and β cell lines are representative for the reactions of normal islet α and β cells, let alone for the reactions of islet α and β cells that have already been exposed to insulitis. In summary, APC-derived TNF-α may be essential in the initiation of insulitis, whereas T cell- and β cell-derived TNF-α may contribute to final β cell destruction.

We described earlier that the conversion from peri-insulitis to destructive insulitis is associated with the production of IFN-γ. The most important sources of IFN-γ in NOD insulitis are $CD4^+$ and $CD8^+$ T cells (37,101,159). Furthermore, NK cells can also contribute to IFN-γ production (101), but we argued before that NK cells do not appear to play an important role in diabetes pathogenesis. *In vitro* studies suggest that IFN-γ may have a direct role in β cell destruction (229). However, *in vivo*, the lack of local (and systemic) IFN-γ, in IFN-γ gene-targeted NOD mice, delays but does not prevent diabetes development (230). Moreover, islets deficient in IFN-γ receptor undergo normal diabetes development in a T cell receptor-transgenic NOD mouse model (218). This suggest that, at least in this model, IFN-γ does not play an essential role in β cell destruction.

It has become clear that cytokines may contribute to β cell damage. The exact mechanism by which cytokines exert cytotoxic effects on β cells has not been clarified completely. *In vitro* experiments suggested that cytokine-induced inducible nitric oxide synthase (iNOS) expression, which generates the free radical nitric oxide (NO) from L-arginine, might be involved (231,232). Alternatively, oxidative stress could play a role.

It is of interest that the kinetics of iNOS expression during spontaneous insulitis in NOD mice closely correlate with those of IFN-γ expression (160,163,165). Also, a correlation between IL-1α mRNA levels and iNOS mRNA levels has been described (165). This suggests that cytokines such as IL-1α and IFN-γ induce iNOS and NO. The *in vivo* treatment of NOD mice with IL-1β, in contrast to treatment with either IFN-γ or TNF-α, leads to iNOS upregulation within the pancreas (233).

The kinetics of NO production not only correlate with those of cytokine production, but also with those of β cell destruction. An enhanced NO production is observed in NOD mouse islets after disease transfer (234). Moreover, levels of iNOS expression in mononuclear leukocytes increase during spontaneous diabetes development (165) and after diabetes acceleration by cyclophosphamide (160). In addition, mRNA levels for iNOS are higher in mononuclear leukocytes from islets of diabetes-prone NOD females than in those from CFA-protected NOD females and NOD males (165).

Functional data also indicate a role for NO in β cell destruction. *In vitro* experiments demonstrate that Mϕ-induced β cell lysis does not occur in the absence of L-arginine, which

is needed for NO production, or in the presence of the iNDS antagonist N(G)-monomethyl-L-arginine (L-NMA) (232,235,236). This indicates that NO is essential for islet cell lysis that is induced *in vitro* in normal islet cells by Mϕ. In contrast, *in vivo*, treatment of NOD mice with the iNOS inhibitors L-NMA or aminoguanidine (AG) has no or only weak effects on spontaneous diabetes development and diabetes transfer (234,237). The weak inhibitory effects of iNOS inhibitors *in vivo* may, however, be related to simultaneous downregulation of the activities of other isoforms of NO synthase, with undefined influences on immune function (237).

Several cells may be the source of NO. Immunohistochemistry shows colocalization of iNOS and the Mϕ-marker F4/80, while no iNOS expression is observed in endocrine cells (238). Other studies localized iNOS in both β cells and Mϕ (165,233). Remarkably, while cytokine-induced NO production is toxic to islets *in vitro*, exogenously supplied NO is not, even at higher concentrations than those produced by islet cells. Based on these observations, it has been suggested that NO production by islet cells themselves is required for cytotoxicity (213). However, islets deficient in iNOS are destroyed with similar kinetics and magnitude as wild-type islets after transfer of diabetogenic $CD4^+$ T cells, indicating that islet iNOS gene expression is not critical in this transfer model (218). This result does not rule out a role for NO produced by endocrine cells during spontaneous diabetes development. Thus, both β cells and Mϕ are sites of iNOS expression during diabetes development.In conclusion, it appears that NO (generated by iNOS), may contribute to β cell destruction.

In vivo studies indicate thatoxidative stress may also play a role. The idea that oxygen free radicals play an essential role in β cell destruction is supported by the finding that transgenic β cell-expression in NOD mice of thioredoxin (TRX), a redox (reduction/oxidation)-active protein that neutralizes oxygen radicals, protects β cells *in vivo* against autoimmune destruction (239). Furthermore, antioxidants protect against diabetes recurrence in islet grafts in NOD mice (240) and prevent spontaneous diabetes development in NOD mice (241). Together, these findings point to a perhaps even more important role for oxygen free radicals than for NO in β cell destruction.

Ultimate β cell death: apoptosis or necrosis?

The final question to be discussed in this context concerns the mechanism of β cell death in the NOD mouse: inflicted cell death as in necrosis or programmed cell death as in apoptosis. Both *in vitro* and *in vivo* studies of β cell death favor the apoptotic mechanism.

In vitro, inflammatory cytokines (IL-1, TNF and IFN-γ) induce apoptosis in the mouse insulinoma cell lines βTC1 and NIT-1 (242,243). Interestingly, the glucagonoma cell line αTC1 shows more resistance to cytokine-induced apoptosis than βTC1 cells, possibly related to higher levels of the anti-apoptotic molecule Bcl-2 (242). It is, however, questionable whether α and β cells that are surrounded by infiltrating cells show the same reactions

as cell lines. For example, apoptosis in response to TNF-α has been observed in NIT-1 cells, whereas apoptosis of primary β cells requires both TNF-α and IFN-γ (244).

Several studies investigated the direct evidence of β cell apoptosis *in vivo*. Islet cell apoptosis as determined by the Tdt-mediated dUTP nick end labeling (TUNEL)-assay is present in NOD mice, but does not colocalize directly with insulin-containing cells (245). Another study described a low frequency of apoptotic cells within the reduced insulin-containing islet area, but the apoptotic phenomena rarely colocalized with β cells (246). Colocalization of apoptosis and insulin-containing cells has been shown in a transgenic T cell receptor NOD-model of rapidly accelerated β cell destruction (247), but it is unclear whether the pathogenic mechanism in this model is representative of spontaneous diabetes development in NOD mice. It is, however, conceivable that the rarity of detectable apoptotic β cells in spontaneous prediabetic mice with pronounced insulitis reflects the relatively long and slow destruction phase in combination with the rapid clearance of apoptotic β cells (246,248). Indeed, apoptotic β cells have been demonstrated after diabetes acceleration via either cyclophosphamide treatment or adoptive transfer (219,246). Apoptosis was, however, not absolutely specific for islet β cells but involved rare α cells as well (219). In conclusion, experimental data suggest that apoptosis is the major mechanism of β cell death, although it can not be excluded that β cells also die by necrosis.

The highway to autoimmune diabetes in NOD mice

In summary, we suggest that the following sequence of events precedes clinical manifestation of diabetes in NOD mice. The first sign of autoimmunity in NOD mice, the accumulation of leukocytes around the islets termed peri-insulitis, is recognizable at about 3 weeks of age. This initiation is mediated by professional APC, including B cells, although B cells are rarely found in histological samples at such early time points. The reasons for the early APC accumulation are not known. Although retroviruses have been found in NOD islets, it is highly unlikely that virus-induced IFN-α expression plays a major role. In these early peri-insulitis stages, $CD8^+$ T cells also appear to be involved. They may cause early β cell death and concomitant release of autoantigens, necessary for the priming and expansion of autoreactive $CD4^+$ T cells. Early infiltrating APC leave the pancreas and present autoantigen to lymphocytes in the pancreas draining lymph nodes. The subsequent migration of these activated lymphocytes into the pancreas causes the formation of HEV and the upregulation of adhesion molecules. Initially, mainly $CD4^+$ T cells are present, of both the Th1 and the Th2 type.

As the insulitis progresses, a shift towards Th1 cytokine profiles takes place. This shift is under the influence of IL-12 and IL-18, which are both exclusively produced by Mϕ and DC. Peri-insulitis progresses to insulitis, in which lymphocytes and Mϕ infiltrate into the islets. Moreover, a progressive decrease in the proportion of $CD4^+$ T cells parallel to an increase in the fraction of $CD8^+$ T cells takes place. Th1-derived IFN-γ induces MHC class I

hyperexpression on islet cells. This facilitates the targeting of MHC class I-restricted $CD8^+$ T cells to islet autoantigens. MHC class II, if expressed by β cells at all, does not play a major pathogenic role in the NOD prediabetes.

In the final β cell killing, two major mechanisms are recognized. The first is the killing of β cells by $CD8^+$ T cells using perforin. The second is killing mediated by the cooperation of $CD4^+$ T cells and Mϕ. In this latter mechanism, TNF-α and IL-1 may be important cell products. Moreover, Mϕ-derived NO and oxygen radicals may play a role. Fas-mediated killing is probably neither involved in killing by $CD8^+$ T cells, nor by $CD4^+$ T cells in conjunction with Mϕ. The ultimate β cell death most likely occurs via apoptosis, although necrosis may also be present. NOD mice typically become diabetic at about 19 weeks of age, illustrating the very long prediabetic period.

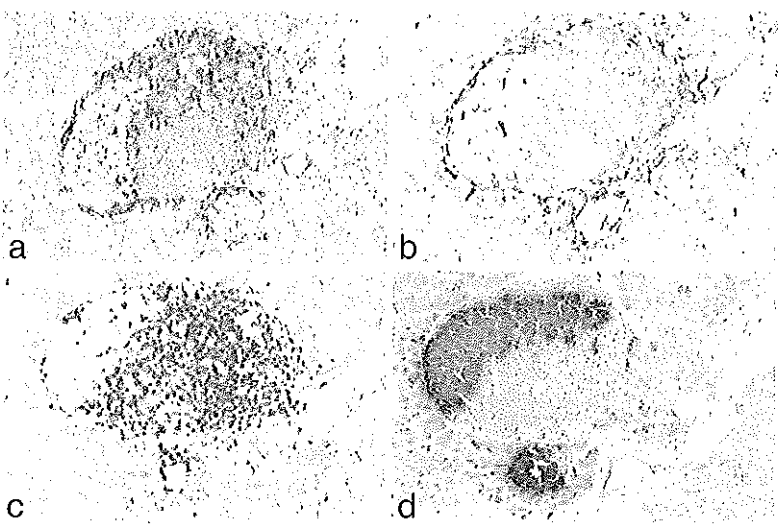

Figure 1: Prediabetic islet infiltrates in a 10-week-old female NOD mouse. (a) $CD11c^+$ DC; (b) $BM8^+$ Mϕ; (c) $CD3^+$ T cells; and (d) anti-insulin.

Figure 1 shows prediabetic infiltrates in a female NOD mouse. The insulin staining (Fig. 1d) shows two islets: a large islet accompanied by a large infiltrate and a small islet that is relatively unaffected. The small islet is still in the initiation phase of infiltration, with accumulated DC (Fig. 1a) and Mϕ (Fig. 1b). The accumulation of T cells is relatively limited (Fig. 1c). Clearly, a large infiltrate is present near the large islet, with many DC (Fig. 1a) and Mϕ (Fig. 1b) and also numerous lymphocytes (Fig. 1c). Despite this massive infiltration, islet function is still preserved, as shown by the anti-insulin staining (Fig. 1d).

BB rat

Autoimmune diabetes in BB rats

The founding animal of the BB rat strain was discovered in an outbred Wistar colony of the BioBreeding Laboratory in Ottawa, Canada (249,250). All BB rats are descendants of the original Ottawa line, but different colonies vary with respect to the frequency and severity of diabetes, probably at least partly due to genetic differences (251). Early in the program of inbreeding, in the fifth generation, animals that failed to develop diabetes were selected to start a control line of nondiabetic BB rats. This line is now designated the diabetes-resistant (DR-)BB rat, in contrast to the diabetes-prone (DP-)BB rat (252). Less than 1% of DR-BB rats spontaneously develop diabetes.

Insulitis in DP-BB rats is evident 7-10 days before diabetes onset. Many autoantigens are involved during disease development (253). About 50-80% of DP-BB rats develop diabetes; the mean age of onset is about 14 weeks. The diabetes incidence in males and females is similar (254). Clinical onset of diabetes is abrupt and characterized by weight loss, hyperglycemia, and elevated levels of circulating ketones and free fatty acids (249). Affected animals die within 2 weeks unless exogenous insulin is given.

DP-BB rats differ from human diabetes patients and NOD mice in that they are severely lymphopenic. The lymphopenia is due to the *lyp* gene, one of the three known gene loci involved in diabetes development in the BB rat. Another important gene locus determining diabetes susceptibility is the MHC (255). The lymphopenia of the DP-BB rat affects all lymphocyte subsets to some degree, but of particular importance is a complete absence of T lymphocytes expressing the RT6 surface alloantigen (256). Normally, RT6 is expressed on ~70% of suppressor/cytotoxic and 50% of helper/inducer T lymphocytes in the rat (257).

In contrast, DR-BB rats are not lymphopenic and have normal numbers of T lymphocytes that express the RT6 surface antigen (258). When a cytotoxic anti-RT6 antibody is injected into 30-day-old DR-BB rats, >50% of RT6-depleted rats becomes diabetic within 4 weeks (259). Similar to spontaneous insulitis in DP-BB, insulitis is evident 7-10 days before diabetes onset in RT6-depleted DR-BB. In contrast, depletion of $RT6^+$ cells in 60-day-old animals fails to produce diabetes. These results suggest that the $RT6^+$ T lymphocyte population in 30-day-old animals is exerting a regulatory influence on the population of effector T lymphocytes responsible for β cell destruction (260). Diabetes can also be induced in DR-BB rats by low-dose irradiation, cyclophosphamide, viral infection, or administration of poly I/C (18).

In addition to insulitis and diabetes, DP-BB rats develop thyroiditis (261) and gastritis (262) and are predisposed to lymphocytic malignancy (263-265). The incidence of associated autoimmunity depends on the subline of DP-BB rats. Moreover, concomitant with the development of severe lymphopenia, DP-BB are susceptible to environmental pathogens (18). The main clinical features of the BB rat model are summarized in Table 1.

In summary, BB rats differ from NOD mice and humans in that they are severely lymphopenic. Moreover, they show a shorter preclinical period during which insulitis is present than NOD mice, suggesting a more progressive insulitis process. In addition, as is the case in humans, the incidence in males and females is similar, whereas in NOD mice females are more diabetes-prone than males.

APC initiate the islet attack

In BB rats, professional APC cells also appear to drive insulitis onset, independent of coincident T cell migration. A summary of findings supporting this notion is given in Table 2.

Several studies have looked at the type of myeloid cells present in normal pancreas and infiltrating the pancreas in BB rats. In the pancreas of control rats, $11F119^+$ DC are detected, mainly in the exocrine tissue. In addition, occasional DC are present in some vessels of the pancreas (47) and a few can be observed in and around the islets (46). In addition, $ED2^+$ (44,46,47) and $ED3^+$ (44,45,47) Mϕ are present predominantly in the exocrine tissue of the pancreas, although they are also observed in some islets (47). One study did not find $ED3^+$ Mϕ at all (46). $ED1^+$ Mϕ are also present, although in much smaller numbers (44).

DC and Mϕ are the predominant cells in islet lesions, both in early stages of spontaneous disease and in diabetes recurrence in islet grafts in DP-BB and RT6-depleted DR-BB (44,45,266,267). When specific stainings to search for the presence of DC are used, it is found that these cells are the majority of early infiltrating cells (46,47). Most initially infiltrating Mϕ appear to be $ED1^+ED2^-$ (44), later an increase in $ED2^+$ and $ED3^+$ cells is found (46,47). $ED2^+$ Mϕ are not likely to be crucially involved in diabetes development in BB rats, since there is no correlation between the distribution of $ED2^+$ cells and the onset of diabetes in individual DP-BB animals (44). In addition, $ED2^+$ cells are not significantly increased in RT6-depleted BB rats as compared to control (267).

Initially, DC and Mϕ are situated around, and in later stages also within the islets in DP-BB rats (46,47). In the RT6-depleted DR-BB rat, it has been observed that, while non-lymphoid cells dominate the insulitis lesion, non-lymphoid cells and T cells tend to enter the islets simultaneously (267). The localization of non-lymphoid cells around the islets may explain the discrepancy between histological studies and flow-cytometric analyses of cell populations present in the infiltrate, since flow-cytometric studies show only low numbers of Mϕ in isolated islets (268).

One study did not show major differences in the number of Mϕ per unit area of exocrine pancreatic tissue between normoglycemic DP-BB and Wistar rats from 70-130 days of age (43), although significant differences with regard to Mϕ phenotype were found. This observation may be taken as an indication that inflammatory Mϕ do not constitute an additional immigrating cell population, but may represent resident tissue Mϕ which have been activated and converted into cytotoxic effector cells.

The cause of myeloid cell infiltration into the islets is unknown, but IFN-α expression by β cells may play a role. IFN-α activates both NK cells (269) and Mϕ (270). IFN-α can already be found in β cells of 30-day-old DP-BB rats, whereas it is never found in DR-BB β cells (53). Moreover, treatment of DR-BB rats with poly I/C induces islet IFN-α expression and diabetes, and similar treatment of young DP-BB rats accelerates diabetes development (53). In line with these findings, anti-IFN-α treatment slightly delays diabetes onset in DP-BB rats (271). Thus, IFN-α may be involved in the initiation of pancreatic APC accumulation. It is speculated that IFN-α expression can lead to the presentation of neo-epitopes, either via an effect on the antigen processing or presentation pathway, or through an effect on the three-dimensional structure of antigens (53). Theoretically, IFN-α expression can be virus-induced. However, both DR-BB and DP-BB rats are maintained in similar specified pathogen free (SPF) conditions. It is thus not likely that viruses can explain the difference in IFN-α expression and subsequent diabetes development between DP-BB and DR-BB rats. The presence of retroviruses can not be excluded, but has not been described to our knowledge.

In summary, in BB rats, as in NOD mice, APC appear to initiate insulitis. The cause of the early APC infiltration may be related to IFN-α expression by β cells in BB rats, whereas IFN-α does not seem to be involved in NOD mice. In BB rats, there are no indications for a viral etiology of diabetes. In NOD mice, the presence of retroviruses has been described, but it is so far unclear whether these viruses are important for the pathogenesis of the disease.

Leukocyte traffic into the pancreas

In the BB rat, little is known about the process of leukocyte extravasation into the pancreas. One study observed mononuclear cell infiltration in DP-BB about 14-21 days before onset of diabetes in peri-vascular sites (44). Also in RT6-depleted DR-BB rats, an accumulation of mononuclear cells at peri-vascular sites in some lobules has been noted during early stages of insulitis (267). Another study detected early peri-vascular infiltration of $ED1^+$ monocyte-like cells in the vicinity of islets following early accumulation of DC around the islets in DP-BB rats (46). It has been suggested that the peri-vascular infiltration of $ED1^+$ cells may represent an early attraction of precursor cells for both DC and Mϕ (46). There are so far no indications for the formation of high endothelium in pancreatic venules, since no morphological changes could be recognized in capillaries of diabetic DP-BB rats in two studies (264,272). To the best of our knowledge, no studies have been reported yet about the involvement of specific adhesion molecules in the BB rat insulitis.

In summary, peri-vascular accumulations, indicating leukocyte recruitment, can be found in BB rats, as is the case in NOD mice. However, in contrast to NOD mice, HEV have not been described. This difference may be related to the relatively minor lymphocyte infiltration in BB rats compared to NOD mice.

The lymphoid actors: major roles for T, but not B and NK cells

In BB rats, lymphoid cells also come into play after the early accumulation of APC. In contrast to what is observed in the NOD mouse model, in which both B and T cells are crucial, in the BB rat only T cells seem to play a prominent role in the pathogenic process. In both the BB rat and the NOD mouse, there is no evidence that NK cells play an important role.

It is highly unlikely that B cells play a major role in diabetes development in the BB rat. B cells are virtually absent in the infiltrates in early stages (44,45). In late stages and in islet grafts in DP-BB rats, they can only be found in low numbers (44,45,266,268), and few plasma cells can be identified among infiltrating B cells (273). One study reported that T and B lymphocytes appear to occupy distinct areas in the infiltrates (273). Also in the RT6-depleted DR-BB, only very few B cells are found in the infiltrates (267). B cells can either function as APC, or mediate β cell destruction via the production of autoantibodies. Autoantibodies reactive with islet cells are indeed present in the majority of animals at weaning, an age at which neither morphological nor metabolic evidence of diabetes is detected yet (274). There is, however, no evidence that these auto-antibodies are pathogenic, since pre-activated B lymphocytes from diabetic DP-BB rats are unable to induce disease upon transfer (276). Moreover, BB rats can become diabetic even when B lymphocytes are depleted from birth (275). Thus, B lymphocytes do not seem to have a crucial role in antigen presentation, nor in β cell destruction during the development of diabetes in the BB rat. In contrast, in the NOD mouse, B cells are essential in antigen presentation, but there is no evidence for a major contribution of B cells to β cell destruction.

In contrast to B cells, T cells are crucially involved in the process leading to diabetes in the BB rat. Despite the profound lymphopenia in the BB rat, significant numbers of T cells are found in infiltrates in both DP-BB and RT6-depleted DR-BB especially in later stages of insulitis (44-47,266,268,277). Insulitis and diabetes can be transferred to young DP-BB rats or immunosuppressed MHC-compatible Wistar rats by mitogen-activated splenocytes of diabetic DP-BB rats (278-281). Conversely, diabetes in DP-BB and in RT6-depleted DR-BB rats is prevented by depletion of T lymphocytes (282-285). Moreover, disease development in DP-BB rats can be prevented by treatments that suppress $CD4^+$ T cytokine production, such as cyclosporin-A (286-288) and FK506 (289,290). Cyclosporin-A also prevents diabetes in RT6-depleted DR-BB rats (291). Together, these findings underline the crucial role of T cells in the development of diabetes in the BB rat.

Only a minority of T cells in early lesions are $OX22^+$, suggesting that the first T cells that arrive belong to the $OX\text{-}22^-$ memory T cell subset. As the disease progresses, naïve $CD45RA^+$ T cells are also recruited into the lesions (267). Both $CD4^+$ T cells (44,45,47,266-268) and $CD8^+$ T cells (45,47,268) are found in DP-BB rats both during spontaneous insulitis and diabetes recurrence in islet grafts and in RT6-depleted DR-BB. The histological

analysis of $CD4^+$ versus $CD8^+$ cells in the rat, however, is complicated by the fact that, in contrast to the situation in the mouse, rat monocytes and Mϕ can express the CD4-antigen, whereas rat NK cells are $CD8^+$.

Functional studies have shed light on the roles of T cell subsets DP-BB and RT6-depleted DR-BB rats. One can hypothesize that $CD4^+$ T cells play a role only in the initiation of insulitis, providing help to $CD8^+$ T cells. In agreement with this hypothesis, recurrent autoimmunity in DP-BB rat recipients of islet transplants can be prevented by treatment with anti-CD8, but not anti-CD4 (292). This suggests that $CD4^+$ T cells are not crucially involved in late phases of β cell destruction in DP-BB rats. Further evidence against a direct cytotoxic role for $CD4^+$ T comes from the absence of MHC class II expression on pancreatic endocrine cells (see later). The observation that transfer of insulitis and diabetes may be accomplished using only the $CD4^+$ splenocyte population from diabetic DP-BB rats (276) can be explained by recruitment of host $CD8^+$ T cells, because adoptive transfer is impossible when either recipients or donors are depleted of $CD8^+$ cells (281). Thus, $CD4^+$ T cells appear to be predominantly involved with initiation of the autoimmune process in BB rats, as is the case in NOD mice.

The role of $CD8^+$ cells in diabetes pathogenesis in BB rats is less obvious. Since both $CD8^+$ T cells and NK cells are $CD8^+$ and most intervention studies will thus affect the function of both cell types, $CD8^+$ T cells and NK cells will be discussed in combination. Whereas NK cells are virtually absent in early insulitis in DP-BB rats (44,45), in late insulitis substantial numbers of NK cells are present (44,45), and NK cells may even be the predominant cell population in late insulitis in DP-BB (268,293). Also in the RT6-depleted DR-BB, NK cells are present predominantly in late insulitis (267,293). However, NK cells in islets of DP-BB are nearly five times more abundant as compared to islets of RT6-depleted DR-BB (293). In RT6-depleted DR-BB rats, $CD8^+$ T cells are reported to be six times more abundant than NK cells.

Based on these observations, early studies have suggested that the critical effectors for β cell destruction may be NK cells for DP-BB and $CD8^+$ T cells for RT6-depleted DR-BB rats. Functional studies, using anti-NK antibodies directed against asialo-GM1, seem to support this hypothesis. DP-BB rat splenocyte cytotoxicity to islets *in vitro* can be blocked by asialo-GM1 treatment *in vitro* (294) or *in vivo* (295). In line with this, anti-asialo-GM1 antibody treatment prevents recurrent autoimmune diabetes in diabetic DP-BB rat islet recipients (267,296). However, asialo-GM1 has turned out also to be present on $CD8^+$ T cells, challenging the interpretation of all studies that used anti-asialo-GM1 to study NK cells (297). When using monoclonal antibody 3.2.3, that recognizes a molecule that is exclusively present on NK cells, NK cell depletion had no effect on spontaneous diabetes development in DP-BB rats (298,299), nor on adoptively transferred diabetes (281). NK depletion with anti-NKR-P1 also had no effect on disease development in both DP-BB and RT6-depleted DR-BB rats (300). In contrast, recurrent autoimmunity in DP-BB rat recipients of islet transplants can

be prevented by treatment with anti-CD8, suggesting an important role for $CD8^+$ T cells in β cell destruction during diabetes development in BB rats (292).

In conclusion, $CD8^+$ T cells play an important role in the final β cell destruction in DP-BB and RT6-depleted DR-BB rats. As in NOD mice, there is no evidence for a major role for NK cells in the development of diabetes in BB rats.

The Th1-Th2 paradigm in insulitis expansion

In the BB rat, early phases of peri-insulitis correlate with a significant expression of both Th1 and Th2 cytokines (301). With the progression of insulitis, Th1 immune reactivity increases in both DP-BB and RT6-depleted DR-BB (301-304). Moreover, islet mononuclear leukocyte levels of IFN-γ mRNA are significantly higher in DP-BB and diabetic rats than in DR-BB rats (303). These studies suggest that destructive infiltrates are associated with Th1 cytokines, whereas non-destructive infiltrates are associated with Th2 cytokines. Indeed, treatments that exacerbate disease correlate with enhanced IFN-γ and decreased IL-10 gene expression (305), whereas protective treatments are associated with the reduction in Th1 and/or the induction of Th2 cytokine production (301,303,306,307). In line with these observations, treatment with antibodies against IFN-γ prevents diabetes in DP-BB (308).

IL-12 is likely to play a role in the shift from Th2 to Th1, since IL-12 mRNA is present both before and during disease onset and levels increase during insulitis in DP-BB and RT6-depleted DR-BB (301,302).

In summary, cytokine expression during the progression of insulitis in both the DP-BB and the RT6-depleted DR-BB rat is comparable to the cytokine expression as observed in the NOD mouse. Early insulitis involves cytokines of the Th1 and the Th2 type; the progression to destructive insulitis is associated with an upregulation of cytokines of the Th1 type. In this conversion to destruction, APC-derived cytokines such as IL-12, and in the NOD mice IL-18, appear to be involved.

The hypothesis of aberrant MHC expression revisited

Several studies have investigated the expression of MHC-peptide complexes during diabetes development in the BB rat. Immunohistochemistry shows endocrine cells to be either negative or very slightly positive in prediabetic DP-BB rats and Wistar controls. However, newly diabetic animals, including DP-BB rats, RT6-depleted DR-BB rats, DP-BB rats after disease transfer and animals with diabetes recurrence after islet grafting, demonstrate markedly enhanced MHC class I staining on endocrine cells (48,266,267,277,309). In addition, MHC class I expression has been quantified by means of mRNA levels, and it has been found that these levels are significantly enhanced in newly diabetic DP-BB rats (277). However, a proportion of the enhancement in MHC class I mRNA is probably attributable to

the high level of MHC class I gene expression in infiltrating inflammatory cells, the remainder representing enhancement within the islet (277). Besides MHC class I overexpression in endocrine cells, enhancement is also seen on ductal epithelium (266,309) and on capillary endothelium (309). Enhanced MHC class I expression is always associated with the presence of inflammatory infiltrate and can be induced by leukocytes in young DP-BB (266,277,309). Interestingly, endothelial MHC class I hyperexpression *in vitro* is only significantly induced by $RT6^-$ T cells isolated from RT6-depleted DR-BB and not by T cells from untreated DR-BB rats (310). In addition, islets from silica-treated animals that do not show lymphocyte infiltration, rarely show MHC class I hyperexpression (48). Together, these observations suggest that MHC class I hyperexpression is a consequence of the inflammatory infiltrate and is not involved in the initial recruitment of inflammatory cells. This MHC class I hyperexpression may be essential for $CD8^+$ T cell-mediated cytotoxicity towards β cells. The kinetics of MHC class I hyperexpression thus follow the same pattern in the BB rat as in the NOD mouse.

As is the case for NOD mice, the induction of MHC class II on BB endocrine cells *in vivo* during diabetes development is controversial. Native endocrine islet cells of several rat strains including DP-BB and DR-BB are MHC class II-negative (266,311,312). IFN-γ is known to induce MHC class II on pancreatic islet endocrine cells *in vitro* (313,314). However, in most *in vivo* studies, MHC class II antigen expression on endocrine cells can either not be observed during spontaneous diabetes and diabetes recurrence in islet grafts (272,277,294,315), or MHC class II is only observed on β cells surrounded by mononuclear infiltrates (309). In some cases, MHC class II^+ β cells are observed in late stages of disease (46,273). However, it is controversial whether β cells are the actual source of this observed MHC class II expression. The interpretation of these histological studies is complicated, since *in vitro* studies on rat islet cells have demonstrated that non-endocrine cells with MHC class II expression can exhibit insulin immunoreactivity in cytoplasmic vacuoles (316). Moreover, it is possible that light microscopy does not have a high enough resolution to distinguish properly between MHC class II on protrusions of DC surrounding β cells, and MHC class II^+ cytomembranes of β cells themselves. Indeed, $MHCII^+$ mononuclear cells dispersed within the islet have been observed, both in normal pancreas (311,315,317) and during the development of diabetes in DP-BB and RT6-depleted DR-BB (48,266,267,272,277). On the RNA level, de novo appearance of transcript homologous to both I-A and I-Eα chain genes in islet RNA preparations from prediabetic DP-BB can be found (277). Since TCR-β chain and IFN-γ gene transcript, which are indices for infiltration, appeared concomitantly, it was concluded that infiltrating cells, and not the endocrine cells, are the major source of MHC class II transcripts (277).

MHC class II can also be induced on non-endocrine pancreatic cell types. Induction of MHC class II on ductal epithelial cells has been found, depending on the strain of the donor (266,315). MHC class II is reported to be absent on normal endothelium (310,311,315,317,318), although one study did find endothelial MHC class II expression

(319). Both induction of MHC class II on islet grafts (266) and the contrary (315) have been described, possibly related to a difference in the antibodies used. Moreover, as is the case for MHC class I, $RT6^-$ T cells isolated from RT6-depleted DR-BB can induce endothelial MHC class II expression *in vitro* (310).

In summary, the data presented here suggest that MHC class II is not induced on endocrine cells in BB rats *in vivo*. Instead, the observed expression may be accounted for by infiltrating mononuclear leukocytes and other non-endocrine pancreatic cells. This is comparable to what is observed in the NOD mouse.

$CD8^+$ T cells and Mϕ are major suspects for β cell destruction

As explained earlier, $CD8^+$ T cells are probably crucial for β cell destruction in both DP-BB and RT6-depleted DR-BB rats. One mechanism by which $CD8^+$ T cells may effect β cell cytotoxicity is the secretion of perforin. In acutely diabetic DP-BB and RT6-depleted DR-BB rats, about 60% of islets contain perforin mRNA, whereas no perforin mRNA-expressing cells can be found in 30 day old non-diabetic DP-BB or non-depleted DR-BB rat islets (293). The number of perforin-expressing cells is comparably low in diabetic DP-BB and RT6-depleted DR BB: about three perforin-expressing cells per positive islet (293). The frequency of perforin-expressing cells is low, but this does not necessarily mean that perforin is not crucial in diabetes development in BB rats. *In vitro* assays have shown that perforin is cytotoxic in a dose-dependent manner to rat islet cells, but not to thyrocytes, indicating a specific susceptibility of β cells for perforin-mediated cytotoxicity (320). It should be stressed, however, that as yet no data exist proving that perforin is cytotoxic to β cells at concentrations that may be present in inflamed islets. To the best of our knowledge, the role of Fas-mediated killing has not been studied in BB rats.

The role of $CD8^+$ T cells in β cell cytotoxicity does not rule out other effector mechanisms. The likelihood that additional cell types may be involved in β cell destruction in BB rats is supported by results from transplantation studies (321,322). After transplantation, MHC-incompatible grafts are significantly less vulnerable to autoimmune attack than MHC-compatible grafts. However, when transplantation is performed in recipients previously tolerized to the graft MHC, both MHC-compatible and MHC-incompatible grafts are equally susceptible to destruction (322). This indicates that MHC-restricted (T cell-mediated) and MHC-non-restricted (Mϕ- mediated) mechanisms may cause β cell destruction. In this respect, the BB rat is comparable to the NOD mouse.

Several *in vivo* and *in vitro* observations support a role for Mϕ in β cell destruction in the BB rat, as is the case in the NOD mouse. *In vitro*, activated Mϕ are cytotoxic to syngeneic rat islet cells and not to cells from other tissues (323). Activated peritoneal Mϕ from normal Wistar and Lewis rats are able to kill freshly isolated rat islet cells (324). Killing was, how-

ever, not β cell-specific and included α and δ cells at the periphery of the islets (324), in contrast to the *in vivo* situation during diabetes development. *In vivo*, a correlation between Mϕ infiltration and β cell destruction is observed, since an attraction of Mϕ can be observed in late insulitis (46,47). Moreover, biopsies show that Mϕ are infiltrating the islets of rats that subsequently develop diabetes, whereas they are low to absent in biopsies from animals that do not progress to diabetes (43). In conclusion, Mϕ may be important effector cells for the final β cell destruction.

Cytokines and free radicals are major β cell destructive weapons

As outlined before, Mϕ are able to lyse islet cells *in vitro*. Electron microscopic studies of cocultures of Mϕ and islet cells have revealed that islet cell lysis is not mediated via a direct contact with Mϕ, but via the release of cytotoxic mediators (235,324,325). Mϕ-mediated injury is probably caused by cytokine release, and the most likely candidates for such destructive cytokines are IL-1, TNF-α and T cell-derived IFN-γ.

IL-1 is cytotoxic to rat β cells *in vitro* (326-336). This effect can be potentiated by adding TNF-α (337,338) and/or T cell-derived IFN-γ (277,339). In line with this, anti-IFN-γ can prevent diabetes in DP-BB by influencing cytotoxicity towards islet cells, but it can also intervene with the disease process via an influence on the Th1/Th2 balance (308).

Besides cytokines, it appears that NO (generated by iNOS) may contribute to β cell destruction in BB rats. Pancreatic iNOS expression correlates with β cell destruction, since it is present in adult DP-BB but not young insulitis-free DP-BB, DR-BB or Wistar rats (340). *In vitro* studies have demonstrated that rat islets are sensitive to the cytotoxic effects of NO (341,342). Accordingly, inhibition of NO-synthesis suppresses the cytotoxicity of activated Mϕ and cytokines against islet cells *in vitro* and is effective in preventing β cell death (235,343-346). *In vivo*, the expression of iNOS is not detected in pancreatic islets without lymphocytic infiltration, nor in Wistar or DR-BB rats, whereas islets with advanced infiltration express iNOS at high levels (340). In addition, inhibitors of NO synthesis have proved to be effective in preventing diabetes *in vivo* (347,348). In conclusion, NO may contribute to β cell destruction.

Several cell types may be responsible for the NO production. iNOS staining is found in areas of $ED1^+$ Mϕ infiltration, although no direct colocalization has been shown (340). Thus, Mϕ themselves appear a likely source of NO. Alternatively, Mϕ may induce adjacent endothelial (349) or endocrine β cells (346,350) to secrete NO by activation via inflammatory cytokines. Cytokines, such as IFN-γ and TNF in conjunction with LPS, appear to inhibit insulin secretion from islets by stimulating the release of IL-1 from intra-islet Mϕ, which subsequently induces the expression of iNOS by β cells (344,346). Exactly the same mechanism has been found for mouse islets.

Stimulation of NO production, however, may not be sufficient for β cell destruction, since cytokine-induced major increases in NO production have been observed that did not affect β cell function (351). Conversely, cytokine-induced β cell changes independent of NO production have also been described (351-353).

Thus, additional factors besides NO are involved in cytokine-mediated β cell destruction. As in NOD mice, it has been suggested that cytokine-induced free oxygen radical formation in β cells is involved in β cells destruction in BB rats (354,355). Indeed, free radical scavengers protect rat islets from cytokine-induced damage *in vitro* (356). Moreover, in DP-BB rats, partial suppression and delayed onset of hyperglycemia are achieved by *in vivo* treatment with free radical scavengers (357).In conclusion, NO formation and oxygen free radicals are likely involved in cytokine-induced β cell damage. Again, in this respect the BB rat shows the same results as the NOD model.

Ultimate β cell death: apoptosis or necrosis?

At least two cellular mechanisms, induced by the events described above, can be responsible for β cell death: necrosis and apoptosis. In isolated rat islets and in rat insulinoma cells, IL-1-induced NO production induces apoptosis (358,359). Moreover, the cytokine combination of IL-1, TNF and IFN-γ also induced apoptosis in rat islets and rat insulinoma cells (243). *In vitro* results are thus in favor of apoptosis, but a contribution of necrosis to β cell death *in vivo* during diabetes development cannot be excluded.

The highway to autoimmune diabetes in BB rats

In BB rats, APC appear to initiate insulitis onset, independent of coincident T cell migration. The initial APC infiltration may be related to IFN-α expression by β cells. The cause of the IFN-α expression is unknown, but it is unlikely that it is virus-induced. After picking up islet-antigens, APC travel to the pancreas draining lymph nodes to present these antigens to lymphocytes. B cells do not appear to participate in this antigen presentation. T cells enter the pancreas, but formation of HEV has not been observed, although peri-vascular accumulations of lymphocytes are present. There is no phase of peri-insulitis; leukocytes immediately start to infiltrate the islets.

B cells can be found in the pancreatic inflammatory infiltrates. However, they do not appear to play an essential role in diabetes pathogenesis. The contrary is true for T cells, despite the profound lymphopenia of the BB rat. Both $CD4^+$ and $CD8^+$ T cells are important, the former mostly in the initiation of insulitis and the latter in β cell destruction. NK cells are present in high numbers within the infiltrates, especially in DP-BB rats, but may nonetheless not be essential for diabetes development. Initially, $CD4^+$ T cells secrete both

Th1 and Th2-type cytokines. Conversion to mainly Th1-type cytokines takes place under influence of IL-12. Pancreatic infiltration upregulates MHC class I on endocrine and exocrine cells, thus facilitating $CD8^+$ T cell-mediated cytotoxicity. Aberrant expression of MHC class II on β cells does probably not occur.

Killing of β cells takes place via different mechanisms. $CD8^+$ T cells exert their actions by perforin-mediated cytotoxicity. It is unknown whether Fas-mediated mechanisms also play a role in $CD8^+$ T cell-mediated β cell destruction. $CD4^+$ T cells together with Mϕ also participate in β cell destruction via the secretion of cytokines and other products. Candidates are the cytokines IFN-γ, TNF-α and IL-1, but NO and oxygen free radicals may also be involved. *In vitro* results favor β cell death by apoptosis, but no *in vivo* evidence has been reported. Diabetes occurs within two weeks after insulitis-initiation; thus the prediabetic phase takes place within a limited time span.

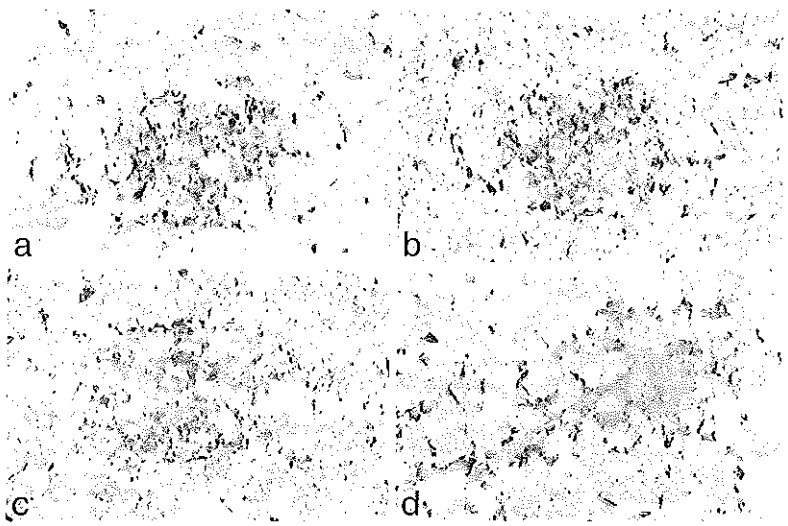

Figure 2: Prediabetic islet infiltrate in a BB rat. (a) $ED1^+$ Mϕ; (b) $ED3^+$ Mϕ; (c) $CD4^+$ T cells; and (d) $RT1B^+$ APC.

Figure 2 shows a representative example of an infiltrate in a DP-BB rat. It can be clearly seen that the infiltrate is small, in contrast to the infiltrates in NOD mice. There is no separate phase of peri-insulitis, inflammatory leukocytes immediately infiltrate into the islet and disturb the islet architecture. Mϕ (Fig. 2a and 2b) and T cells (Fig. 2c for $CD4^+$ T cells) are present in equal numbers, in contrast to the situation in NOD mice, where T cells constitute the majority of the infiltrate. Also, numerous APC are present throughout the infiltrate (Fig. 2d). These infiltrates in the BB rat are relatively aggressive, since the prediabetic phase is only 7-10 days in BB rats, as compared to many weeks in NOD mice.

Human type 1 diabetes

Type 1 diabetes in humans

Before reviewing human diabetes pathogenesis, we wish to underline a concern that should be kept in mind when interpreting findings related to human diabetes pathogenesis. As stated before, symptoms of diabetes in humans do not develop until the majority of pancreatic β cells is destroyed. The disease process therefore will have been initiated long before its clinical manifestation (360). This is evidenced by the fact that, during this preclinical period, autoantibodies are circulating, T cell responses to β cell antigens can be found and changes in the response to glucose challenge are evident (1-3). Most studies concerning autoimmune processes occurring in the pancreas in human type 1 diabetes use autopsy material. It should therefore be stressed that these observations inevitably represent end-stage disease manifestations. Thus, limited information is available about the initiation phase in human diabetes. Moreover, only few reports are available concerning the expansion phase of human insulitis. These reports have been published during the entire last century. Techniques employed in these studies thus vary considerably, and make these studies difficult to compare. Finally, as outlined below, it is very likely that human type 1 diabetes patients are heterogeneous in the causes of and maybe the processes leading to the disease. All these factors complicate research on processes leading tot type 1 diabetes in humans.

APC initiate the islet attack?

In human type 1 diabetes, the role of Mϕ and DC in the initiation of insulitis is less clear than in the animal models due to the virtual impossibility of studying the initiation phase. However, indirect evidence suggests that myeloid cells are involved. In normal pancreas, a small but significant number of myeloid cells has been found located among acini and ducts (93). Mϕ and DC are also observed in pancreatic infiltrates in virtually all type 1 diabetes patients (93,214,361-365). In one case, no monocytes or Mϕ could be found in the infiltrate using antibodies against CD11c and CD14 (366). Overall, Mϕ in pancreatic infiltrates tend to be fewer in number than lymphocytes in most cases of diabetes (93,95,214,363-365,367,368), although a predominance of Mϕ over lymphocytes has also been described (95,364). This does not, however, rule out a predominant influx of Mϕ and DC at the initiation of insulitis, since these cases represent end-stage disease.

The reason for the initiation of autoimmunity is unclear. Several studies suggest that virus-induced IFN-α expression by islet cells is involved. IFN-α has been found in endocrine cells in a proportion of islets in diabetic (54,369) and prediabetic (369) pancreases. In one of these studies, only β cells stain strongly (54). Another study shows that IFN-α and to lesser

extent IFN-β and IL-6 are also present in control pancreases, but, in contrast to diabetic pancreases, in no case the combination (93). Furthermore, a significant increase in the level of IFN-α mRNA has been found in the pancreas of diabetes patients as compared with controls (52). Endocrine IFN-α expression may result in local changes in expression of MHC and adhesion molecules. However, during viral pancreatitis, no clear correlation between IFN-α expression by β cells and the level of expression of MHC class I by adjacent cells has been found (54).

The exact kinetics of IFN-α expression during human insulitis are not clear, due to the scarcity of prediabetic material. It is, however, unlikely that the expression of IFN-α is entirely due to local inflammation, since IFN-α expression does not occur in other situations with massive leukocyte accumulation in the pancreas, such as chronic pancreatitis or graft-versus-host-disease (54). Also, the disturbances in glucose homeostasis are an unlikely cause of IFN-α expression, since pancreatic IFN-α expression is not observed in type 2 diabetes (54). Only in viral pancreatitis comparable IFN-α expression has been found, lending support to the hypothesis of a viral etiology for human type 1 diabetes (54). Also the combination of IFN-α, IFN-β and IL-6 expression, that has been found in diabetic pancreases, can be explained by a viral infection (93).

Coxsackie B enteroviruses are the most obvious candidates for such an infection. Coxsackie virus-specific IgM responses and viral RNA are more common in recent onset patients than in controls (370-372). Moreover, Coxsackie B virus, cultured from a diabetic pancreas, has been able to cause diabetes in normal mice (373). In addition, Coxsackie B viruses cause endocrine cell necrosis in the human pancreas (374,375). Besides Coxsackie B enteroviruses, mumps virus, cytomegalovirus and congenital rubella may be involved in some cases of diabetes (376-379). Moreover, the infection of human insulinoma cells with measles, mumps and rubella virus induces the expression of cytokines (notably, IL-1 and IL-6) and upregulates MHC molecules (380), which can make them more prone to autoimmune attack.

In contrast to these studies, other studies have failed to reveal any evidence of infection by the candidate viruses in collected autopsy diabetic pancreases (93,381,382). It is especially important to note that there have been no reports in the last 20 years of a virus cultured from an autopsy pancreas of a patient with recent-onset type 1 diabetes. Moreover, although there is a marked tropism of Coxsackie viruses for the endocrine pancreas and inflammatory changes accompany islet infection, these changes are qualitatively different from the ones found in recent onset type 1 diabetes. Especially the presence of numerous granulocytes after Coxsackie infection (382) represents a completely different situation from the prediabetic inflammatory process, since only a few polymorphs are found in most diabetic autopsy pancreases (383-388). However, two cases have been reported in which an extensive infiltration of polymorphonuclear leukocytes was observed (389). In one of these cases, an acute diffuse pancreatitis is seen, with necrosis of some of the large ducts and an extensive infiltration of polymorphs in the interstitial tissue, especially involving the interlobular tissue. In these last two cases, an acute virus infection may have caused diabetes directly. However, a direct

cytolytic effect of viruses is less likely to be compatible with the long pre-clinical period that precedes diabetes in most patients. It should be realized that a β cell reduction up to 80% can be compensated for by the remaining β cells. Virus infections or stress may then lead to a sudden collapse of insulin secretion. A virus or anti-viral IgM being detected at diabetes onset does therefore not prove that type 1 diabetes is of viral origin, since diabetes development has presumably been initiated long before its clinical manifestation (390).

In conclusion, the evidence is against a direct cytolytic effect for viruses resulting in diabetes. It remains possible that viruses that infect the pancreas cause subclinical β cell damage. The resulting release of autoantigens and cytokines within the pancreas may break tolerance towards β cell autoantigens and ultimately lead to type 1 diabetes (391). The observation that maternal enterovirus infections are a risk factor for childhood diabetes in offspring may fit into such a scenario (392-394). Viruses can, however, also act as initiators of autoimmunity via the presence of molecular mimicry: structural similarities between viral and β cell proteins. Functional cross-reactivity for T cells has been described for an epitope shared by the major diabetes autoantigen GAD and the Coxsackie B P2C protein. Moreover, an IA-2 epitope has been reported to show sequence similarity (but not identity) with VP7, a major immunogenic protein of human rotavirus and with epitopes from other viruses that have been implicated in the pathogenesis of type 1 diabetes (395). Several other cross-reactivities between viral and β cell proteins have been described (396-398). However, with emerging knowledge of human and viral and microbial DNA sequences, the chances of finding sequence similarities are increasing. Such a hit may be coincidental until functional cross-reactivity has been demonstrated. Moreover, most studies are based on similarities in the primary protein structure, neglecting the fact that the tertiary structure may be of higher physiological significance. In addition, direct evidence supporting the role of mimicry in the pathogenesis has never been provided.

Finally, viruses may also initiate type 1 diabetes by the activation of autoreactive T cells by a retroviral superantigen. The enrichment of Vβ7-carrying T cells in recent onset diabetes patients, the subsequent cloning of a superantigen encoded by HERV-K10 (*IDDMK$_{1,2}$22*) and the demonstration that this virus mediates a Vβ7-biased superantigen effect on lymphocytes has led to the hypothesis that retrovirally encoded superantigens are involved in type 1 diabetes (399,400). However, later studies have consistently shown that this possibility is not likely, given the lack of difference in *IDDMK$_{1,2}$22* expression and in seroreactivity to the *IDDMK$_{1,2}$22* protein between patients and controls (401-407). Furthermore, the putative Vβ7-specific stimulation of the *IDDM$_{1,2}$22* protein has also been contradicted (403).

In summary, APC may initiate insulitis in humans, as is the case in NOD mice and BB rats, although there is no direct evidence for such a scenario. The initial APC infiltration can be related to IFN-α expression by β cells in humans, like in BB rats but in contrast to NOD mice. In most human diabetes cases and in animal diabetes, an acute viral infection does not seem to be involved in the endocrine IFN-α expression and initiation of diabetes. The pres-

ence of retroviral particles has only been described in NOD mice, but it is so far unclear whether these viruses are important for diabetes development in these mice. In humans, no conclusive evidence for a retroviral initiation of type 1 diabetes has been found.

Leukocyte traffic into the pancreas: highways of adhesion molecules

It is conceivable that the pancreas-infiltrating APC pick up antigens and present them to T cells in the pancreas-draining lymph nodes. These T cells can subsequently home to the islets, using adhesion molecules. In two studies, peri-vascular infiltrates were observed in autopsy pancreases (84,408). Local differentiation of the endothelium toward a more HEV-like morphology was found in both cases, with a parallel accumulation of mononuclear cells around those vessels. Since high endothelium is never detected in venules devoid of peri-vascular infiltrates, and since not all venules with peri-vascular infiltrates display HEV morphology (408), it seems reasonable to assume that the differentiation towards HEV-morphology is the result rather than the cause of peri-vascular infiltration. These findings suggest that such venules are sites of active extravasation in vivo (84).

The formation of HEV is comparable to the NOD mouse and contrasts with the situation in the BB rat, where HEV formation has not been observed.

In human type 1 diabetes, it is difficult to define the exact order in which adhesion molecules are implicated in leukocyte extravasation into the pancreas. Several adhesion molecules (summarized in Table 3) are thought to be involved in the development of diabetes, since their expression patterns change during disease development. It should be realized that the differences between normal and diabetic pancreas may be due to the diabetic state per se, since the expression of adhesion molecules is known to be influenced by glucose levels (409).

In normal pancreas, ICAM-1 is expressed on capillary endothelium and epithelium of some ducts (93). In autopsy diabetic pancreases, expression of ICAM-1 is increased on endothelial cells in islets (93-95) as well as in many blood vessels in the exocrine pancreas (94). This increase in ICAM-1 expression is found in association with islets with insulitis and residual insulin content (93). Since LFA-1 is expressed on infiltrating cells, ICAM-1/LFA-1 is at least one pathway by which endothelial cells may control inflammatory cell influx into the pancreas in a non-tissue specific manner (93). The ICAM-1/LFA-1 adhesion pair might also play a role in antigen presentation and in β cell cytotoxicity. Infiltrating cells remain ICAM-1 negative (94,95), so bi-directional LFA-1/ICAM-1 interactions on APC and T lymphocytes are probably not involved in enhancing local antigen presentation during insulitis. Several studies have considered a role for the ICAM-1/LFA-1 interaction in β cell cytotoxicity, since induction of ICAM-1 on endocrine cells in vitro by proinflammatory cytokines (TNF-α and IFN-γ) has been reported (96,97). However, as is the case in normal pancreas (96), islet cells are always ICAM-1 negative in diabetic pancreas (94,95), thus making a role for ICAM-1 in β cell cytotoxicity unlikely.

LFA-3 and its T cell-restricted ligand CD2 are suggested to be responsible for the co-stimulating activity provided by non-professional APC, such as endothelial cells and β cells, in antigen presentation experiments (410). The expression of LFA-3 on pancreatic endothelial cells is controversial; it was reported to be increased in endothelial cells of one patient (95), while another study showed comparable staining to control pancreas (93). More consistency exists regarding β cell expression: LFA-3 was absent from normal β cells (96) and islet cells of a recent onset diabetes patient (95) and was not inducible on islet cells by proinflammatory cytokines *in vitro* (96). In conclusion, LFA-3 is probably not crucial in antigen presentation by non-professional APC during human diabetes development.

In contrast to what has been found in the NOD mouse, VCAM-1 is not expressed on endothelium in islets or in exocrine areas of the diabetic pancreas or control pancreases (93,94), making a role of VCAM-1/VLA-4 interactions in leukocyte extravasation unlikely. However, VCAM-1/VLA-4 interactions may be involved at another level, since in normal pancreas, a small number of VCAM-1^+ DC-like cells can be found (93). Moreover, in normal pancreas, VLA4 is expressed on epithelium and walls of ducts and vessels (93). It is therefore possible that the VCAM-1/VLA-4 interaction is involved in DC extravasation into the pancreas and the islets. The role of VCAM-1 in human diabetes development needs further clarification.

PNAd expression has not been observed in endothelial cells in diabetic pancreas, suggesting that the PNAd/L-selectin pathway is not necessarily functioning in human diabetic pancreas (84). Anti-E-selectin also did not stain any venules in diabetic or control pancreases (94).

In conclusion, similarities and differences exist in adhesion pathways between insulitis developing in humans and in NOD mice, whereas adhesion pathways have not been studied in detail in BB rats. In the NOD, the MAdCAM-1/LPAM-1 interaction, PNAd/L-selectin and ICAM-CR3 are involved in early phases of leukocyte extravasation. In humans, in contrast, the PNAd/L-selectin pathway does not appear to be involved, whereas the other two have not been studied. In both NOD and humans, the ICAM-1/LFA-1 interaction is probably important, in humans mainly in leukocyte extravasation, in NOD mice also in antigen presentation. In both humans and NOD mice, ICAM-1/LFA-1 interactions are probably not involved in β cell destruction. In NOD mice, but not in humans, the VCAM-1/VLA-4 pathway also appears to play a role in leukocyte extravasation.

The lymphoid actors: major roles for T, and probably B and NK cells

Lymphoid cells play a prominent role in the development of type 1 diabetes in humans. In normal human pancreas, a small but consistent number of lymphocytes can be found among acini and ducts (93). Lymphocytes are observed in all diabetes autopsy pancreases with insulitis, and are thought to play important roles in human diabetes

development. NK cells have not been extensively studied in human diabetes. In one study, NK cells were reported to be absent in human insulitis (93).

Considering the presence of B cells in the infiltrates, an extensive amount of variability exists. B cells are present in some cases (94,95,361,364,366,367,387) and absent in others (93,95,363). Usually, B cell numbers are lower than T cell numbers, although two reports describe almost equal numbers of T and B cells (94,364). Only in few patients, B lymphocytes predominate (364). Plasma cells are usually not observed (93,366,383-385,387). In two cases, organized accumulations of lymphoid cells have been found, with germinal center formation in one (383) and clusters of B lymphocytes in the other (361). Also, HEV and $RFD1^+$ interdigitating cells are found in the T cell zones (361).

B cells may either be involved as APC, or mature into autoantibody-secreting plasma cells. These autoantibodies may be involved in β cell destruction, although variable results have been obtained regarding the deposition of autoantibodies in the diabetic pancreas. Autoantibody deposition on islet cells was not found in several patients (93,363,411), although scarce and interstitial immunoglobulin deposits could be found in one of these cases (93). Also arguing against the involvement of autoantibodies in the destruction is the observation that the serum of one of these patients failed to stain his own viable islets (93). In contrast, an abnormal deposition of IgG on the outer membrane of endocrine cells was found in another study (366). Functional studies suggest that autoantibodies may contribute to diabetes development in some patients. Islet-cell-surface-antibody $(ICSA)^+$ sera from diabetes patients caused significant lysis of cultured rat islet cells in the presence of complement, whereas $ICSA^-$ sera did not (412). In general, the evidence is nonetheless against a pathogenic role for auto-antibodies in human diabetes development. Despite transplacental transfer of maternal auto-antibodies, children with diabetic mothers have a lower chance of developing diabetes than children with diabetic fathers (7). Furthermore, transplacental transfer of maternal antibodies does not lead to an accelerated onset of type 1 diabetes in those children who will eventually develop type 1 diabetes (M. Knip, personal communication). Moreover, human B lymphocytes producing diabetes-associated auto-antibody do not cause islet damage upon transfer to (complement-competent) *scid* mice (413).

The same variability as observed for autoantibody involvement applies to the involvement of complement. Contrasting reports exist on the deposition of complement components in the pancreas. In some patients, no deposition of component C3 or the membrane attack complex on islet cells was found (93,363). In another patient, however, a proportion of cells in all sections showed abnormal depositions of C9 (366), indicating that in this patient, complement-mediated islet cell lysis may have occurred. In general, type 1 diabetes is not associated with complement activation (414).

In conclusion, autoantibodies in some human patients may contribute to β cell destruction. In the majority, however, they do not seem to play a pathogenic role. This is similar to the situation in BB rats and NOD mice. In the latter, this may be related to a complement-

deficiency. Whether B cells are involved as APC, as observed in NOD mice, but not the BB rat, is unclear for human diabetes.

T cells in pancreatic infiltrates have been studied extensively and are present in all cases of human diabetes. At the onset of diabetes, T cells are abundant among islet-infiltrating cells. The same observation has been made during diabetes recurrence in diabetes patients transplanted with pancreas isografts or HLA-identical allografts (362,363,415). Indications for the essential role of T cells come from the observation that cyclosporin treatment prolongs endogenous insulin production (416,417). Moreover, antibodies directed against T cells (anti-thymocyte globulin) have positive effects on metabolic control, although only short-term (418). In addition, recurrent autoimmunity in islet transplants can be prevented if the immunosuppressive therapy includes anti-thymocyte globulin (419). The essential role of T cells in human diabetes is thus comparable to their role in both NOD mice and BB rats.

Less consensus exists about the subtype of T cells present in the inflammatory infiltrates. The predominant T cell in the pancreatic infiltrates is in most cases $CD8^+$ (93-95,362,363,366), in agreement with a role for $CD8^+$ T cells in late stages of insulitis. However, a predominance of $CD4^+$ T cells in insulitis has also been observed (214,361). In addition, a human $CD4^+$ T cell clone has been described which lyses MHC class II-compatible islet cells (420), suggesting that $CD4^+$ T cells are able to destroy β cells by direct cytotoxicity.

These observations are in contrast to observations made in animal models. Whereas $CD8^+$ T cells are more abundant than $CD4^+$ T cells in human insulitis, in both BB rats and NOD mice $CD8^+$ T cells are present in lower numbers than $CD4^+$ T cells. This may suggest that $CD8^+$ T cell cytotoxicity is more prominently involved in human type 1 diabetes than in the animal models. In both BB rats and NOD mice, however, $CD8^+$ cells do appear to play important roles in diabetes development. Moreover, the human data represent end-stage insulitis, and in late stages in NOD mice, the accumulation of $CD8^+$ T cells is also increased. In some human cases, $CD4^+$ T cells are present in higher numbers than $CD8^+$ T cells. These cases thus follow the kinetics observed in the animal models.

The Th1-Th2 paradigm in insulitis expansion

Also in human diabetes, indications exist for a selective involvement of Th1 cells in islet destruction. IFN-γ mRNA is expressed at high levels in the pancreas of diabetes patients, while IL-4 mRNA is lacking (421), suggesting a dominance of Th1 cells in pancreatic infiltrates. In accordance with this, IFN-γ is expressed *in situ* by 40% of the lymphocytes during diabetes and 45% during prediabetes (365). Furthermore, β cell antigen responses of antigen-specific T cell lines generated from diabetes patients have been shown to be associated with IFN-γ and TNF-α production, suggestive of a Th1-like phenotype (422). In addition, all T

cell clones of $CD4^-CD8^-$ $V\alpha24J\alpha Q^+$ T cells, which are presumed to promote Th2 rather than Th1 bias, that were isolated from diabetes patients secreted only IFN-γ upon stimulation. In contrast, almost all clones from the at risk non-progressors and controls secreted both IL-4 and IFN-γ. It was therefore suggested that Th1 cell mediated tissue damage is initially regulated by T cells producing both Th1 and Th2 cytokines and that the loss of the capacity to secrete IL-4 is correlated with type 1 diabetes (423). The transition to an almost exclusively Th1 type infiltrate possibly involves IL-12. In conjunction with this, levels of this cytokine are significantly higher in islet cell antigen $(ICA)^+$ relatives of diabetes patients than in controls (350). The initial presence of both Th2 and Th1 cytokines, and the conversion to a Th1-type destructive infiltrate under influence of IL-12 can thus be found in human diabetes patients, BB rats and NOD mice.

Not all studies are in agreement with a Th1/Th2 disbalance in pancreatic infiltrates. One study did not find any T cell cytokines, including IL-2, IL-4, IL-10 and IFN-γ, in pancreas tissue using RT-PCR (93). Another study was not able to detect TNF-α or IL-2 mRNA in control or diabetes pancreases, and found highly variable expression of IL-1β, IL-4 and IL-6 mRNA with no pattern emerging from the comparison of the four diabetic and the four non-diabetic individuals (52). These discrepancies probably reflect heterogeneity in human diabetes pathogenesis.

The hypothesis of aberrant MHC expression revisited

Also in humans, several studies have compared the expression of MHC molecules in diabetic pancreas to that in normal pancreas. In normal pancreas, expression of MHC class I and MHC class II is found on capillary endothelium (93,424,425). Furthermore, MHC class I and occasionally MHC class II is found on ductal cells (93,424,425). Endocrine cells in normal pancreas are either negative or weakly positive for MHC class I and MHC class II (93,424,425). MHC class II has also been found in islets on rare DC-like cells (93).

In diabetic autopsy pancreases, an increased expression of MHC class I is found on endothelial cells within and around islets (93,95,411,426) and on ductal epithelium (93). Furthermore, all studies find a marked increase in MHC class I expression on islet cells in a proportion of islets in diabetes patients (93-95,366,369,411,425,426) and also in a prediabetic individual (369). Some studies report the endocrine MHC class I hyperexpression to be independent of the presence of lymphocytic infiltration (366,369), others report MHC class I hyperexpression preferentially in islets with insulitis (93,95,365). This discrepancy may be explained by the observations that infiltrates can be quite focal and may thus be easily missed. Alternatively, one study showed that all insulin-containing islets with insulitis overexpressed MHC class I, while this was not necessarily true for inflamed insulin-deficient islets. However, MHC class I hyperexpression also occurred in non-inflamed insulin-containing islets from diabetes patients (425). These kinetics suggest that MHC class I hyperexpression precedes insulitis, and ceases again after the destruction of β cells. This complex relationship

between insulitis and MHC class I hyperexpression may explain the contrasting findings about the association of insulitis and MHC class I hyperexpression.

MHC class I hyperexpression is not only observed in β cells, but also in α and δ cells (94,411,425). Most MHC class I hyperexpressing islets contain residual β cells (54,369,425), suggesting that β cells in those islets are secreting a substance that has a paracrine effect inducing hyperexpression of MHC class I on adjacent endocrine cells. Candidate for such a substance is IFN-α, and indeed a strong correlation between IFNα expression and MHC class I hyperexpression is found (54,369). However, during viral pancreatitis, no clear correlation between IFN-α expression by β cells and the degree of expression of MHC class I by adjacent cells could be found (54), arguing against IFN-α as sole determinant for MHC class I hyperexpression. In addition, IFN-γ produced by infiltrating cells can cause islet endocrine cells to express MHC class I at elevated levels (427,428). However, during chronic pancreatitis, MHC class I hyperexpression is not observed, although comparable numbers of IFN-γ producing T cells are present as found in diabetic autopsy pancreases (365), arguing against an essential role for IFN-γ in MHC class I hyperexpression.

In conclusion, MHC class I hyperexpression by endocrine cells is present in humans, comparable to BB rats and NOD mice. However, in humans it may precede insulitis, whereas MHC class I hyperexpression is induced by infiltrating cells in the animal models.

As expected, MHC class II expression is found in the mononuclear cell infiltrate of inflamed islets (94,361-363,366-368,387). Enhanced MHC class II reactivity is also observed on vascular endothelium (94,95,362,363,366,368,387,411,426). Variable results have been obtained with regard to the expression of MHC class II on endocrine cells. MHC class II is reported to be either negative on endocrine cells (94,362,367,387) or positive specifically on a fraction of β cells in some patients (93,366,411,425). Since only a fraction of β cells hyperexpress MHCII, their presence may be missed because of a limited localization. Alternatively, the observed islet MHC class II expression may be accounted to by intra-islet leukocytes, as is the case in animal diabetes. When MHC class II is seen on endocrine cells, islets expressing MHC class II without evidence for insulitis are also observed (368,425,429). This observation has led to the suggestion that on aberrant expression of MHC class II on β cells precedes insulitis, similar to the kinetics of MHC class I expression, and is therefore not entirely induced by the inflammatory infiltrate. Although a focal inflammatory infiltrate may be missed, it is indeed unlikely that inflammatory cytokines are entirely responsible for a positive endocrine MHC class II expression. Aberrant MHC class II expression is absent on β cells in pancreatic inflammatory diseases (425). Moreover, inflammatory mediators in *in vitro* studies failed to induce MHC class II expression selectively on β cells, but instead also induced MHC class II expression on other pancreatic endocrine cells (427,428,430,431). Finally, pro-inflammatory cytokines such as IFN-γ may not be able to cause human β cells to express MHC class II *in vivo* at all, given the observation that diabetes recurrence after transplantation of an identical twin pancreas did not involve MHC class II expression by β cells, whereas IFN-γ was significantly expressed (363).

In conclusion, intra-islet MHC class II expression has been observed in the human diabetic pancreas. However, it has not been convincingly shown that intra-islet MHC class II is expressed on endocrine cells, as opposed to intra-islet leukocytes. If MHC class II is aberrantly expressed on endocrine islet cells, inflammatory cytokines are not likely to be the only cause. In BB rats and NOD mice, MHC class II molecules are most likely not induced at all on endocrine cells.

An alternative explanation for the expression of MHC class I and MHC class II molecules on islet cells comes from the observation that normal ductal cells express chromogranin, C-peptide, glutamic acid decarboxylase (GAD) and unexpectedly MHC class I and MHC class II (93). Neogenesis of islets from ductal cells is typical of acute onset type 1 diabetes and probably represents an attempt to replace the destroyed β cells. The MHC-expressing cells found in recent onset human type 1 diabetes may thus represent regenerating β cells. In accordance with this, the ability of IFN-γ and TNF-α to induce MHC class II on β cells is related to the differentiation state of the β cell (431). In conclusion, although MHC expression is also present in islets not affected by insulitis, it could be a consequence of the inflammatory process rather than a cause.

$CD8^+$ T cells and Mϕ are major suspects for β cell destruction

In human type 1 diabetes development, the composition of the insulitis appears to be heterogeneous. For example, in some patients $CD8^+$ T cells predominate, whereas in others $CD8^+$ T cells or B cells comprise the major lymphocyte population. It is conceivable that these differences in cell involvement in the insulitis process also result in different non-mutually exclusive β cell killing mechanisms. Indeed, indications have been found for several possible killing strategies. It has been discussed before that autoantibody- and/or complement-mediated β cell killing mechanisms may play a role in diabetes development, in contrast to what is observed in animal models. In addition to these pathways, $CD8^+$ T cells and Mϕ contribute to β cell destruction in human type 1 diabetes patients.

In one study, human $CD8^+$ T cells were shown to specifically inhibit insulin release by mouse islet cells cultured *in vitro* (432), but the interpretation of these data is complicated by the absence of MHC restriction in this model. In addition, prediabetic twins have elevated levels of $HLA\text{-}DR^+CD8^+$ T cells as compared to control subjects and low-risk twins (433). Since these cells have the same cytotoxic phenotype as the predominant cell type found in the islets at diagnosis, the circulating cells may have a role in the pathogenesis of islet damage (433). $CD8^+$ T cells may cause β cell damage by the release of pore-forming molecules, such as perforin, and by Fas-mediated apoptosis induction.

Variable results have been obtained concerning the expression of perforin in diabetic autopsy pancreas. In one case, $CD8^+$ T cell cytotoxicity was supported by detection of per-

forin transcripts (93). In another case, however, perforin was not found in infiltrating cells (369). Thus, in this case, cytotoxicity mediated by perforin was not playing a major role in β cell destruction, but Fas-induced apoptosis could have been the major β cell killing mechanism (369).

Normal islet cells do not express Fas (434-436), but normal β cells do express FasL constitutively as shown by Western blot (434). This situation seems to differ from that in rodents, where FasL expression is reportedly absent from the endocrine pancreas under normal circumstances. However, immunohistochemical studies also failed to detect FasL expression in humans (214,436). IL-1 increases Fas-expression and apoptosis in normal β cells stimulated by anti-Fas. Normally, anti-Fas does not induce apoptosis because of a lack of Fas-expression of normal β cells (214,434,435). It has been suggested that a selective expression of Fas in β cells, primed by IL-1β-induced NO, may be responsible for their specific killing as T cells expressing FasL may promote an MHC unrestricted destruction of Fas^+ bystander β cells, while sparing neighboring Fas^- α and δ cells (214). Thus, after *in vitro* cytokine treatment, human islet cells are primed to undergo apoptosis by the simultaneous presence of both Fas and FasL. *In vivo*, a clear correlation has been found between Fas-expression and insulitis in biopsy specimens of recent onset type1 diabetes patients, suggesting that inflammatory cytokines secreted by islet-infiltrating cells induce Fas expression on endocrine cells (436). In one diabetes patient, extensive apoptosis among Fas^+ β cells located in proximity to FasL-expressing T lymphocytes infiltrating the islets was observed (214). However, another study described 6 patients with Fas^+ β cells and $FasL^+$ T cells, with no evidence of islet cell apoptosis (436). It has been suggested that this absence of apoptosis can be explained by the very short term of completion of apoptosis.

In conclusion, in some human diabetes patients Fas/FasL-induced apoptosis may be involved in β cell death. In NOD mice, Fas/FasL interactions are probably not crucial for diabetes development, whereas these interactions have not been studied in the BB rat. The mechanisms of β cell destruction, as observed in animal models, may therefore not be representative for all human patients. In humans, functional Fas expression is induced on β cells by NO. In contrast, NO has no effect on either a Fas expression or Fas-mediated apoptosis of NOD mouse islet cells. This suggests a species difference in the effects of cytokines and cell-death-inducing agents on islet cells.

Besides $CD8^+$ T cell-mediated cytotoxicity, $CD4^+$ T cells or products of T cells and Mϕ, such as IL-1, TNF and IFN-γ, appear to play the main role in β cell destruction. It should, however, be realized that cytokine-induced islet cell destruction is not selective for β cells, but involves other endocrine islet cells as well (437).

Cytokines and free radicals are major β cell destructive weapons

TNF-α, IL-1 and IFN-γ are important cytokines for β cell destruction. Evidence for an *in vivo* role of both TNF and IL-1 comes from a study in identical twins of diabetes patients. In twins who subsequently developed diabetes, levels of circulating TNF-α and IL-1α are elevated above the normal range more frequently than in those who do not (438). In newly diagnosed type 1 diabetes patients, an increase of TNF-α (438-442), IL-1α (438) and IFN-γ (438) plasma levels can be observed. Further evidence supporting an *in vivo* role for IFN-γ comes from the detection of IFN-γ in diabetes autopsy pancreases (365,443).

In vitro studies suggest a mechanism by which these cytokines can cooperate. IL-1 is cytotoxic to β cells in cultured human islets (332,334,437), an effect that can be amplified by adding TNF-α (444) and/or IFN-γ (437). It is suggested that resident islet Mϕ, activated by inflammatory stimuli such as cytokines and cellular damage, may inflict β cell damage by releasing IL-1 in human islets (445).

The role of NO in β cell-destruction during human diabetes development has not been resolved yet. Several studies support a role for NO in cytokine-mediated β cell destruction. Cultured human islets are susceptible to the destructive effects of NO (341,446). In addition, cytokine-induced changes in β cell function can be inhibited by iNOS inhibitors (445,447).

In contrast, other studies suggest that cytokine-induced NO production in human islet cells may be neither necessary nor sufficient to destroy islet β cells. Cytokine-induced β cell destruction independent of NO has been described (434,448-451). Moreover, cytokine-induced iNOS expression and nitrite formation that were not associated with effects on the function of cultured human islets have been observed (450,451). Therefore, other mediators of cytotoxic cytokine effects on human islets are also involved. As is the case for BB rats and NOD mice, oxygen free radicals may play a role.

The role of NO in cytokine-induced β cell damage has been compared with that of oxygen free radicals in one *in vitro* study (448). The combination of IL-1β, TNF-α and IFN-γ induces significant increases in malondialdehyde (MDA), an end product of lipid peroxidation, and nitrite, and significant decreases in insulin and DNA in islets. These changes can be inhibited by an antioxidant but not by the iNOS-inhibitor L-NMA, although the latter does prevent cytokine-induced nitrite production. These results suggest that cytokines may be toxic to human islet β cells by inducing oxygen free radicals, which cause lipid peroxidation and aldehyde production in the islets (448). Indications for a role for oxidative damage to β cells *in vivo* have also been found. The total plasma antioxidant status is significantly lowered in ICA^+ subjects when compared to both ICA^- and healthy subjects, while no significant difference is found between ICA^+ subjects and recently diagnosed diabetes patients (452). In conclusion, oxygen free radicals can also mediate cytokine-induced β cell destruction.

In summary, the same cytokines, IL-1, TNF-α and IFN-γ, are suggested to be implicated in β cell death in human islets and in NOD mice and BB rats. They may work via the

induction of NO production or oxygen free radicals. However, *in vitro* cytokine-induced iNOS expression in human islet cells differs from that in rodent islet cells. While iNOS mRNA and NO production is induced by IL-1β alone in rodent insulin-producing cells, a combination of two (IL-1β and IFN-γ) or three (+ TNF-α) cytokines is required for iNOS activation in human pancreatic islets (453). Moreover, addition of cytokine combinations to isolated rodent islets can lead to the destruction of β cells within 24h (454,455); in human islet cell preparations, the cytotoxic effect is first noticed after more than 3 days of exposure (451). Thus, rodent islet cells appear more sensitive to cytokine-induced β cell destruction than human islet cells.

Ultimate β cell death: apoptosis or necrosis?

The final question is whether human β cell death is achieved via necrosis or apoptosis. The fact that cytokines (IL-1β,TNF-α,IFN-γ) exert β cell cytotoxicity *in vitro* by inducing apoptosis favors the latter option (434,451). One *in situ* study reported an extensive apoptosis among Fas^+ β cells located in proximity to FasL expressing T lymphocytes infiltrating the islets (214).

In addition to apoptosis, necrosis may also occur in some cases. *In vitro*, β cell damage caused by peroxynitrite occurs via necrosis (446). In a diabetes autopsy pancreas, no apoptotic cells were observed by EM (93), although this may be due to the low frequency of apoptotic cells in a disease with a long pre-clinical period. In conclusion, both β cell apoptosis and necrosis may be involved in human type 1 diabetes.

The highway to type 1 diabetes in humans

The summary of the pathogenesis of type 1 diabetes in humans is somewhat more complicated than in NOD mice and BB rats, since virtually nothing is known about the early events responsible for the initiation of insulitis. Viral infections are not likely to cause acute-onset diabetes, but may result in subclinical β cell damage, that could trigger autoimmunity. In recent-onset diabetes, an expression of IFN-α by islet cells can be found, which may trigger an immune response. The subsequent extravasation of leukocytes into the pancreas leads to the formation of pancreatic HEV and the upregulation of adhesion molecules on vascular and endocrine cells.

Accumulated lymphocytes consist of both T and B cells; especially the amount of B cells varies between patients. It is not likely that B cells are major β cell destructors by means of autoantibody production. The predominant T cell within the infiltrates is $CD8^+$. The evidence suggests that $CD4^+$ T cells initially produce Th1 and Th2 cytokines. A shift to Th1 cytokines takes place in conjunction with the appearance of destructive insulitis. This transition may be related to the presence of IL-12. MHC class I hyperexpression on islet cells can

make them easy targets for $CD8^+$ T cell cytotoxicity. It is not likely that this hyperexpression is entirely the result of cytokines (notably IFN-γ) produced by infiltrating cells, as it may well be present before the initiation of insulitis. These same kinetics apply to the expression of intra-islet MHC class II, although it has not been convincingly shown that endocrine cells are the source of intra-islet MHC class II expression.

Final β cell destruction involves both $CD8^+$ T cells, using FasL and perforin to induce cytolysis, and $CD4^+$ T cells in conjunction with Mϕ. The Mϕ products involved in the latter mechanism include TNF, IL-1, IFN-γ, NO and oxygen free radicals. The major effector mechanisms may differ between individuals. Both apoptosis and necrosis lead to final β cell death.

Comparing human and animal diabetes pathogenesis

In contrast to experimental animals, human type 1 diabetes patients are heterogeneous with regard to genetics and environmental factors influencing diabetes development. It can thus be argued that each of these animal diabetes models represents a small subgroup of type 1 diabetes patients. However, with regard to the pathogenesis of the disease, the NOD mouse and the BB rat exhibit remarkable similarities. It is conceivable that the etiology of autoimmune diabetes development may be different between these experimental animals, but the mechanisms leading to disease are comparable. The same may apply for type 1 diabetes developing in humans: although the cause of the disease may differ between individual patients and between patients and animals, the routes leading to disease are remarkably similar. Therefore, we will discuss a bird's-eye view of diabetes pathogenesis, and the exceptions found in humans and experimental animals. Figure 3 represents a putative general scheme for the pathogenesis of type 1 diabetes and its animal model, based on the evidence provided in this review.

The initiation of insulitis is dependent on the influx into the pancreas and/or homing to the islet periphery of DC and Mϕ in both NOD mouse and BB rat. In humans, this may be similar, but this is difficult to prove. The reason for this initial accumulation of potential APC is not known, but has been suggested to be related to the presence of IFN-α in both humans and BB rats, but not in NOD mice. In both humans and BB rats, it is highly unlikely that this IFN-α expression is caused by viral infection. In these early phases, $CD8^+$ T cells are also required for the expansion of autoreactive $CD4^+$ T cells in both NOD mice and BB rats.

Early infiltrating APC presumably pick up antigens in the pancreas to present them in the pancreas draining lymph nodes to lymphocytes. In this antigen presentation, B cells appear to play a crucial role in the NOD mouse but not in the BB rat, although it should be realized that the role of B cells has been more extensively studied in the former than in the latter. Leukocytes enter the pancreas via several different adhesion molecule pairs.

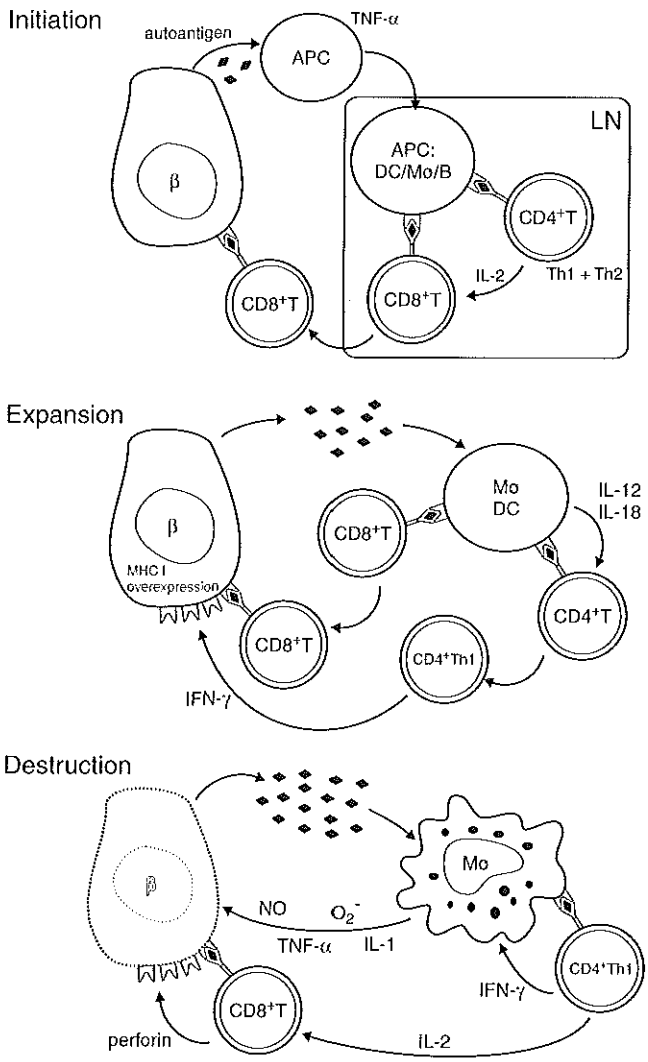

Figure 3: Putative general scheme for the pathogenesis of type 1 diabetes and its animal models.

In both animal models and human diabetes, T cells play a crucial role in the expansion of the insulitis. These T cells in animal and human diabetes initially produce both Th2 and Th1 cytokines. As soon as they lose their capability to produce Th2 cytokines, the infiltrates become destructive for β cells. In this conversion to destruction, the APC-derived cytokine IL-12 plays a role in humans, BB rats and NOD mice. In NOD mice, IL-18 is also important; the role of this cytokine is not studied yet in BB rats and human type 1 diabetes patients.

In both animal and human diabetes, MHC class I overexpression on endocrine cells is seen. Endocrine MHC class II expression is not present in the BB rat and the NOD mouse, while the source of intra-islet MHC class II expression in human type 1 diabetes patients has not been convincingly determined. MHC (over)expression may facilitate β cell killing.

Final β cell destruction can follow different pathways, partly dependent on the diabetes model. Autoantibodies appear not to be pathogenic in animal models, although they may be in some patients. Direct cytotoxicity by $CD4^+$ T cells is also not likely to play a role in the majority of the cases. The major destruction mechanisms in animal and human diabetes are direct cytotoxicity by $CD8^+$ T cells and $CD4^+$/Mϕ DTH reactions resulting in local production of IL-1, TNF and IFN-γ. Pore-forming molecules such as perforin are involved in $CD8^+$ T cell-mediated cytotoxicity in NOD mice, BB rats and human diabetes patients. Fas seems not essential in β cell killing in the NOD mouse, but may be involved in some human patients. In humans, functional Fas expression is induced on β cells by NO. In contrast, NO has no effect on either Fas expression or Fas-mediated apoptosis of NOD mouse islet cells. This suggests a species difference in the effects of cytokines and cell-death-inducing agents on islet cells.

Cytokine-mediated β cell destruction in human and animal diabetes probably involves the production of NO and oxygen radicals by β cells themselves and/or the infiltrating inflammatory cells, in particular activated Mϕ. However, rodent islet cells appear to be more sensitive to cytokine-induced β cell destruction than human islet cells. *In vitro* studies on islet death should, however, be interpreted with caution. The fact that cytokines are able to kill β cells, does not mean that they are also actually important in β cell killing *in vivo*. Moreover, the normal islets used in these *in vitro* studies may not be representative for the responses of islets that are already affected by an inflammatory process. The final β cell death occurs most likely via apoptosis, but necrosis may also be involved.

Diabetes research today faces major challenges. With the increasing incidence of type 1 diabetes, the need for prevention strategies also increases. More knowledge about the initiation phase of diabetes is essential for prevention of the disease. Moreover, the possibility to identify individuals at risk for diabetes obliges us to search for strategies to inhibit an ongoing, but not yet fully developed pathology. Therefore, knowledge of the development of insulitis and the initiation of destruction is essential and should be expanded. Finally, the ultimate goal of diabetes research is to cure those that are already affected. For this purpose, a β cell could possibly be engineered that is resistant to recurrent diabetes and can thus be transplanted into diabetic patients. In this case, it is essential to have a thorough knowledge of the relevance of the possible killing strategies in order to prevent them. Experimental animals, in particular the BB rat and the NOD mouse, are useful to study prevention, but essential to investigate potential abrogation and reversal of developing human type 1 diabetes.

In 1901, Opie was the first one to note a connection between damage to the islets of Langerhans and the development of diabetes. Almost 100 years later, the exact mechanisms

that cause islet damage have started to be unraveled, mainly because of the use of experimental animals. Insight into these mechanisms is of major importance for diabetes research. In our opinion, a continued effort of parallel research into human and experimental animal diabetes eventually will bring cure for this disease to reality.

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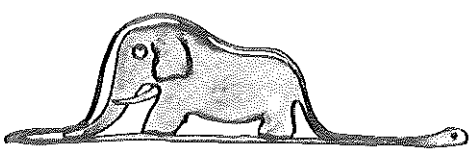
Chapter 1.2

T cell education during diabetes development in humans, BB rats and NOD mice: teachers and students



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The immune system has evolved to protect the body against a multitude of harmful intruders. At the same time, an effective immune response to the own body is blocked; we are self-tolerant. Two important mechanisms deal with T cell tolerance. Central tolerance induction during T cell development in the thymus ensures the deletion of autoreactive T cells. Peripheral tolerance induction ensures that potentially self-reactive T cells, which escaped central deletion, are not harmful to the body. However, T cell tolerance induction sometimes fails, as is the case in T cell mediated autoimmune diseases. Type 1 diabetes mellitus (type 1 diabetes) is a classical example of a T cell mediated autoimmune disease. Several aberrations in immune regulation, which may have consequences for tolerance induction, have been described in both human diabetes patients and in animal models for autoimmune diabetes. In this review, we summarize how proposed immune defects may be implicated in the loss of T cell tolerance towards self in human and animal autoimmune diabetes, in particular in the NOD mouse and the BB rat. For this purpose, we will discuss the tolerance-inducing mechanisms that an autoreactive T cell should encounter from is genesis to its pathogenesis in order of appearance.

Central tolerance

Central T cell tolerance is effectuated during thymic development of T cells. The specialized environment of the thymus ensures that a useful, non-damaging repertoire of T cells is generated from BM-derived pro-thymocytes. For this purpose, the entire spectrum of T cells is selected for those cells recognizing peptides in the context of MHC with sufficient affinity (positive selection) but with limited affinity for self-peptides associated with MHC molecules (negative selection). Classically, it is thought that the cortical epithelial network mediates positive selection whereas BM-derived DC and epithelial cells in the thymic medulla play a role in the deletion process of negative selection. Thus, the question arises whether dysfunctional T cell selection in the thymus plays a role in the development of autoimmune diabetes. Indeed, several defects in central tolerance induction have been found to be associated with diabetes development (Table 1).

Table 1: Defects that might hamper positive and negative selection during central tolerance induction in NOD mice (N), BB rats (B) and humans (H) (for references see text).

	Thymic epithelium	Thymic APC	Thymocytes
Positive selection	• Selection-bias towards high-affinity CD4+ T cells because of poor peptide binding of diabetes-associated MHC class II haplotypes (N, H) • lack of positive selection of regulatory T cells (N)		
Negative selection	• disturbed reticulum structure with loss of corticomedullary junction that is essential for negative selection (N) • large reticulum-free areas without MHC hamper negative selection (N,B) • lack of negative selection of autoreactive CD4+ T cells related to diabetes-associated MHC class II haplotype (N)	• defects in antigen degradation lead to suboptimal antigen processing (N,H) • lack of negative selection of autoreactive T cells related to diabetes-associated MHC haplotype (N)	• hyporesponsiveness to TCR-stimulation leads to disturbed tolerance induction (N) • disturbed apoptosis induction (N) • genetic link towards apoptosis-associated molecules (H) • increased apoptosis rate (B)

Positive selection in the thymus of diabetes-prone individuals biases for high affinity T cells and a lack of regulatory T cells

The purpose of positive selection is to generate a T cell repertoire that is able to recognize antigenic peptides in the context of self-MHC. Positive selection depends on the lymphostromal interaction of developing thymocytes with the thymic cortical epithelial stroma. In this process, the newly rearranged TCR complex expressed on developing $CD4^+CD8^+$ thymocytes interacts with MHC expressed on cortical thymic epithelium (TE).

It has been shown that TE has a crucial role in the development of diabetes. Grafting of purified TE from NOD embryos to newborn C57BL/6 nude mice results in the development of insulitis (1). Thus, T cell education by the NOD thymic epithelium is sufficient to induce autoimmune infiltration in the pancreas of normal mice. Given that epithelium has a unique function in mediating positive selection that can not be mediated by bone marrow-derived cells, such as DC (2), these findings suggest that an anomaly in thymic positive selection of T cells contributes to diabetes development in NOD mice. The exact identity of this anomaly in thymic positive selection is at present unknown. Since grafted TE appears to induce tolerance in allogeneic recipients because it selects for regulatory $CD4^+$ T cells (3), it has been suggested that NOD TE fails to select an appropriate repertoire of regulatory T cells. This aberrant function of the NOD TE may relate to unique diabetes-associated MHC molecules, which genes are the primary genetic components that determine susceptibility to diabetes in both humans and experimental animals. A possible role for MHC expressed on TE in the hampered positive selection of regulatory T cells has been studied by making NOD mice diabetes-resistant by the transgenic expression of a protective MHC class II haplotype. These studies indicate that protective MHC class II alleles may indeed act through positive selection of regulatory T cells (4,5). Thus, TE expressing diabetes-associated MHC molecules may fail to positively select regulatory T cells.

In addition to this lack of selection of regulatory T cells, diabetes-associated MHC class II molecules expressed on TE may induce positive selection that is biased towards high-affinity T cells. Diabetes-associated MHC class II molecules have special features compared to protective ones; the most obvious one is the expression of a non-Asp amino acid at position 57 of the β chain. Approximately 85-90% of type 1 diabetes patients under the age of 17 is homozygous for expression of the DQβ 57 non-Asp haplotype; susceptibility is also associated with a lack of Asp at position 57 in the DRβ chain (6). Similarly, NOD mice are homozygous for a non-Asp amino acid, serine, at position 57 of the β chain of the murine counterpart of DQ, $I\text{-}A^{g7}$ (7). Noteworthy, the requirement of a non-Asp amino acid at β chain position 57 for diabetes development is not observed in the BB rat. However, also in the BB rat, an essential role in the development of diabetes has been found for genes mapping to the MHC class II region of the $RT1^u$ haplotype (8). Studies in NOD mice have indicated that diabetes-associated MHC haplotypes may positively select for high-affinity T cells. The diabetes-associated MHC class II haplotypes $I\text{-}A^{g7}$ in NOD mice (9) and HLA-DQ3.2 in humans (10) appear to bind peptides with low avidity. Results of several groups suggest

that unstable I-A^{g7}-peptide complexes produce an effectively decreased density of MHC-peptide complexes on thymic APC. This reduced MHC class II expression would positively select for a population of T cells with high TCR affinity to attain the avidity threshold required in thymic positive selection (11).

In summary, NOD TE expressing diabetes-associated MHC molecules positively selects for a T cell repertoire with a bias towards high-affinity T cells and a lack of regulatory T cells.

Negative selection in the thymus of diabetes-prone individuals results in a high frequency of autoreactive T cells, due to an aberrant organization of TE, antigen processing defects and thymocyte apoptosis defects

The purpose of negative selection is to generate a repertoire of T cell specificities that lacks autoreactive cells. In this process, TCR on developing T cells interact with MHC-peptide complexes on TE and/or BM-derived APC, resulting in the deletion of self-reactive T cells.

BM-derived APC, in particular thymic medullary DC, play an important role in the negative selection process. Several defects in the function of BM-derived APC have been found in human and animal diabetes, as will be discussed further in this review. It is often suggested that these defects may impair thymic negative selection of high affinity T cells. However, it should be realized that it is not clear to which extent adult peripheral blood monocyte-derived DC functionally mirror thymic DC. In fact, the composition of the thymic APC population differs from that of peripheral BM-derived APC (12). Apart from BM-derived APC, medullary TE can mediate clonal deletion and thus negative selection. Major defects have been found in thymic stroma in animal diabetes. In NOD mice, the normal structure of the thymus is disturbed, since subtypes of epithelial cells that are normally restricted to the medulla are found in the NOD thymic cortex as well (13). Repositioning of medullary epithelial cells may implicate that T cells are undergoing negative selection processes at an abnormal stage of T cell development. This aberrant timing of T cell education may result in a loss of tolerance, since the choice between tolerance and immunity depends on the state of differentiation of the lymphocyte at the time it encounters its cognate antigen (14). Disorganization of the thymic epithelium also leads to the loss of the corticomedullary junction prior to the development of diabetes (15). The corticomedullary junction is thought to represent the anatomic location of negative selection and, as a consequence, disturbances in this region may hamper negative selection (15). In addition, large reticulum-free areas are found in thymuses of both NOD mice and BB rats; these areas are devoid of epithelial cells and MHC expression (13,16-18). Interestingly, the size of reticulum-free areas is larger in thymi of diabetes-prone BB rats compared to thymi of diabetes-resistant BB rats (17). Studies using transgenic, chimeric and KO mice have shown that tolerization of autoreactive thymocytes requires appropriate expression of MHC molecules by medullary stromal cells (15). Since

optimal anatomical organization of thymic medullary stroma with an appropriate expression of MHC molecules is required for tolerance induction, the described aberrations in TE organization may predispose to the development of autoimmunity.

Besides their disturbed thymic expression, the structure of diabetes-associated MHC molecules in itself may affect negative selection processes. Studies using NOD mice double transgenic for an I-A^{g7}-restricted diabetogenic TCR and a diabetes-resistant MHC class II haplotype indicate that protective MHC alleles may act through negative selection of autoreactive T cells (19). Moreover, additional differences are present between protective and susceptible MHC class II alleles. Peptide-binding studies indicate that susceptible MHC class II molecules present different epitopes of the major autoantigen GAD65 to T cells compared to resistant MHC class II molecules (20). Cross-immunization experiments using peptides revealed that the protective GAD-epitopes were immunogenic in animals with susceptible alleles. However, a panel of epitope-specific I-A^{g7} restricted T cell hybridomas obtained from these animals respond only to GAD65 peptide, but not to the whole protein, suggesting that antigen processing differences exist between mice with different MHC class II alleles (20). In summary, unique diabetes-associated MHC class II molecules may fail to mediate deletion of autoreactive $CD4^+$ T cells.

Antigen processing defects have been suggested to affect the negative selection of autoreactive $CD8^+$ T cells. These defects involve the MHC-linked genes Tap1 and Tap2, both of which encode proteins that contribute to MHC class I processing (21-23). However, also defects in the function of the proteasome may affect antigen processing and MHC class I presentation in NOD mice and human type 1 diabetes patients. In general, two major forms of the proteasome exist: the standard proteasome, consisting of the catalytic subunits β1, β2 and β5, and the immunoproteasome, consisting of the subunits LMP2 (β1i), MECL1 (β2i) and LMP7 (β5i). Immunoproteasomes are induced by IFN-γ and may be constitutively expressed in mature DC (24). The immunoproteasome is more efficient than the standard proteasome in processing a number of antigenic peptides (25). Leukocytes of humans with type 1 diabetes and NOD mice appear to be disturbed in the production of the LMP2 β subunit of the immunoproteasome (21,23,26). The reduced LMP2 activity is associated with an altered substrate specificity of immunoproteasomes (21,23). In the thymus, the reduced LMP2 activity may thus directly interfere with the preparation of peptides for MHC class I education of $CD8^+$ T cells, and both the NOD mouse and diabetic human exhibit partial ablation of this pathway (27). Thus, defects in the expression of proteins involved in antigen processing, such as Tap1, Tap2 and LMP2, may result in an inappropriate preparation of peptides for MHC class I-mediated education and thus in an inadequate negative selection of $CD8^+$ T cells.

Finally, defects in the thymocytes themselves contribute to an inadequate negative selection in the thymus. Negative selection in diabetes-prone animals appears to be hampered by both hyporesponsiveness of thymocytes upon TCR stimulation, and by impaired apoptosis induction in thymocytes. Thymocytes from NOD mice respond weakly to anti-CD3-induced proliferation, compared with thymocytes from several control mice (28). Defective TCR-mediated cell death in activated NOD thymocytes appears to be associated with a block

in Ras activation and defective signaling along the PKC/Ras/MAPK pathway (29). The block in Ras activation is accompanied proximally by an impaired recruitment of TCR-CD3-ξ-associated tyrosine kinase ZAP70 to membrane-bound TCRξ (30). Interestingly, this impaired membrane-recruitment of ZAP70 may have implications for apoptosis induction, since both TCRξ and membrane-localized ZAP-70 are required for the upregulation of FasL. Accordingly, T cells that display impaired recruitment of ZAP-70 in the plasma membrane will be unable to undergo Fas-mediated apoptosis and negative selection will be impaired (31). Thus, aberrations in the TCR-signaling pathway in NOD mice may impair apoptosis induction during negative selection.

Several studies indicate that apoptosis defects are indeed present in NOD mice and human diabetes patients. Functionally, NOD thymocytes exhibit a genetically determined resistance to the induction of apoptosis by a variety of stimuli (32-34). This may be related to an upregulation of the anti-apoptotic protein Bcl-x on NOD thymocytes (34). The contribution of defects in thymocyte apoptosis induction to the loss of tolerance during human diabetes development is hard to test experimentally. However, genetic studies indicate a role for apoptosis-related molecules in human diabetes development. The apoptosis-associated molecule Fas-associated death domain protein (FADD)/MORT1 is candidate for diabetes susceptibility region IDDM4 (35) and CTLA-4 for IDDM 12 (36). It is conceivable that these genetic defects may influence thymic negative selection. In contrast to humans and NOD mice, thymocytes in BB rats display an increased apoptosis rate, resulting in a profound deficiency in peripheral T cells (37-39). Analysis of peripheral lymphocyte populations in thymus and BM chimeras revealed that the abnormal BB thymus is not responsible for the immunodeficiency, but that the lymphopenia defect resides within the lymphocyte precursor pool (40). The increased death rate in BB thymocytes is at least in part the result of the *lymphopenia (lyp)* mutation, although also other genes in the BB rat appear to be involved (39).

In summary, negative selection in diabetes-prone individuals is hampered by defects in all participating cells. The organization of the TE is disturbed, and large areas devoid of MHC expression can be found in animals prone to develop diabetes. The role of defects in thymic bone marrow-derived APC clearly deserves further attention. Furthermore, the unique diabetes-associated MHC class II molecules may fail to negatively select autoreactive $CD4^+$ T cells. Meanwhile, abnormalities in proteins involved in MHC class I processing negatively affect the preparation of peptides for the clonal deletion of autoreactive $CD8^+$ T cells. Finally, defects in thymocytes themselves contributing to inadequate negative selection include both hyporesponsiveness of thymocytes upon TCR stimulation and impaired apoptosis induction in thymocytes. The impaired negative selection in the thymus likely results in a high frequency of autoreactive T cells in the periphery. NOD mice indeed contain a high number of autoreactive T cells in the periphery, as has been demonstrated by the possibility to break peripheral tolerance to self-peptides (41) and by limiting dilution assays (42). This increased frequency of autoreactive peripheral T cells may strongly interfere with peripheral tolerance, since one of the major factors that reverses indifference and leads to autoimmunity is an increase in the frequency of autoreactive T cells (14).

Peripheral tolerance

The outcome of central tolerance induction in diabetes-prone individuals is a T cell repertoire that is skewed towards high-affinity autoreactive T cells. Peripheral tolerance mechanisms should prevent activation of these cells in the periphery. However, several mechanisms operative in peripheral tolerance induction show defects that further contribute to the loss of self-tolerance leading to diabetes development. These defects include a hampered induction of activation-induced cell death (AICD), resistance towards cytokine withdrawal death and a functional deficiency of regulatory T cells (summarized in Table 2).

Table 2: Defects that may hamper mechanisms of peripheral tolerance induction in NOD mice (N), BB rats (B) and humans (H) (for references see text).

	Peripheral APC	T cells
AICD	• Immature APC with antigen processing defects result in poor T cell stimulation, thus hampering AICD (N,B,H) • Constitutive PGS_2 expression leads to inhibition of T cell IL-2 signal transduction (N,B,H)	• TCR signaling defects may result in disturbed apoptosis induction (N,H)
Cytokine withdrawal death		• T cells aberrantly survive severe IL-2 deprivation (N,H)
Regulatory T cells	• Aberrant APC maturation results in poor stimulation of regulatory T cells (N,B,H) • Absence of I-E shifts Th1/Th2 cytokine balance in direction of Th1 (N)	• Absence of regulatory NKT cells in poor stimulation of regulatory (N,B,H) • *Lyp* mutation results in absence of regulatory $RT6^+$ T cells (B)

AICD: severely hampered by a combination of constitutive APC PGS_2 expression and T cell apoptosis defects

AICD is the result of the interaction between peripheral APC and T cells. AICD is achieved upon APC-mediated TCR stimulation accompanied by simultaneous ligation of death receptors such as Fas and other TNF receptor family members (43).

APC defects may predispose to autoimmunity through quantitative reduction in signals required for AICD, since activation-driven T cell death requires quantitatively more stimulation than antigen-driven T cell proliferation (44). Several studies suggest that APC in diabetes-prone individuals may be unable to fulfill the T cell stimulation requirements for AICD.

An aberrant Mϕ and DC maturation, putatively resulting in an impaired APC function, is associated with diabetes in NOD mice, BB rats and humans. NOD BM-derived progenitor cells of granulocytes and monocytes proliferate poorly in response to several myeloid growth factors, including IL-3, GM-CSF and IL-5 as compared to progenitor cells derived from normal mice (26,45,46). Several studies suggest that these defects in myelopoiesis in NOD mice may impair both Mϕ (45-48) and DC (49) maturation. The immaturity of splenic DC freshly isolated from BB rats is evidenced phenotypically by a low MHC class II and CD80 expression and functionally by a relatively high phagocytic activity as well as poor T cell stimulation capacity (50). Also human diabetes patients and high-risk relatives show disturbances in APC maturation as they have an impaired yield and function of blood monocyte-derived DC (51,52). Moreover, expression of CD80 and CD86, but not HLA-DR, is decreased on DC from high-risk relatives of type 1 diabetes patients (51). Thus, it appears that Mϕ and DC maturation is disturbed in human type 1 diabetes as well as its animal models. The observed APC immaturity might hamper AICD, since immature APC have been recognized as relative poor T cell stimulators as compared to mature and activated APC (53,54).

Several studies suggest that also other features of APC in diabetes-prone individuals may interfere with the relatively high APC stimulus that is required for AICD. A decreased ability to process antigens can contribute to less effective antigen presentation. NOD mouse splenic Mϕ have a lower concentration of intracellular glutathione compared to diabetes-resistant NOR mice. Since glutathione is involved in the degradation of antigens, these low levels result in less efficient processing and lower levels of T cell activation (55). The lack of immunoproteasomes in diabetes-prone individuals, discussed before, may further contribute to impaired processing and thus to suboptimal T cell activation and the lack of AICD. In summary, various types of antigen processing defects in APC probably disturb the induction of AICD because these defects result in a suboptimal T cell stimulation.

In addition to maturational abnormalities and antigen processing defects, an aberrant constitutive prostaglandin synthase 2 (PGS_2) expression in APC may influence AICD induction in human diabetes patients, BB rats and NOD mice. Mϕ from NOD mice and BB rats are able to produce high levels of immunosuppressive factors, such as prostaglandins (PG) and leukotriens (56,57). Constitutive expression of the normally inducible enzyme PGS_2 in Mϕ leads to an enhanced prostanoid metabolism in female NOD mice (58). A similar aberrant constitutive expression of PGS_2 is found in monocytes of patients with type 1 diabetes and humans at increased risk of developing diabetes (58), and thus seems to be a general phenomenon occurring during diabetes development. The aberrant PGS_2 expression that is found during diabetes development may directly hamper AICD, since PG are able to suppress T cell IL-2 signal transduction which is, in turn, critical for the activation of T cells and thus for AICD (58). These findings suggest that a constitutive PGS_2 expression and in consequence a high PG production in APC of diabetes-prone individuals hampers AICD via an inhibition of T cell IL-2 signaling.

AICD may also be disturbed due to abnormalities in signal-transduction pathways in the T cells themselves. Patterns of unresponsiveness and decreased cytokine secretion have been observed in anti-TCR/CD3-activated NOD peripheral T cells, similar to what was found in NOD thymocytes (59). If peripheral T cells show the same signaling defects as described for thymocytes, these defects may result in the same apoptosis disturbances. In line with this, NOD peripheral lymphocytes have been found to exhibit a genetically determined resistance to the induction of apoptosis by several stimuli (32,34,60-63). Accordingly, these T cells are relatively resistant to undergo AICD. Also in recent onset diabetes patients, a defect in the signal transduction pathway of the TcR/CD3 complex was shown, resulting in ineffective PKC activation and an abnormal IL-2 production and proliferation of diabetic T cells (64). Thus, similar to the signaling defects described in NOD mice, human TCR signaling defects may hamper apoptosis induction during AICD. Because of the important role of Fas in AICD, it is worth noting here that the apoptosis-associated molecule Fas-associated death domain protein (FADD)/MORT1 is candidate for the human diabetes susceptibility region IDDM4 (35), and that the expression of Fas is highly impaired on leukocytes from type 1 diabetes patients (65).

Together, these data indicate that AICD-mediated peripheral tolerance induction in diabetes-prone individuals is affected at the APC side by immature PGS_2-expressing APC with antigen processing defects, resulting in suboptimal stimulation of T cells. At the T cell side, TCR signaling defects and a resistance to apoptosis induction further contribute to the hampered AICD.

Cytokine withdrawal death: NOD and human activated T cells survive severe IL-2 deprivation

Cytokine withdrawal death may serve to remove the expanded population of lymphocytes after an antigen has been cleared successfully during a regular immune response (43). It may thus prevent autoimmune destruction caused by bystander-activation of autoreactive T cells. This tolerance mechanism also is affected, as activated T cells from human diabetes patients require 20-100 times less IL-2 than normal T cells to escape apoptosis, and thus survive severe IL-2 deprivation (66). Similarly, activated lymphocytes from NOD mice exhibit a similarly increased resistance to apoptosis following IL-2 withdrawal associated with an upregulation of the anti-apoptotic Bcl-x protein in activated T cells (34). Taken together, the increased resistance of lymphocytes in diabetes-prone individuals to apoptosis induced by growth factor withdrawal facilitates the survival of emerging autoreactive clones.

Regulatory T cells: Absence of certain regulatory T cell subsets, whereas immature I-E$^-$ APC fail to stimulate other regulatory T cell subsets

Regulatory T cells control autoreactive T cells that have escaped both central and peripheral tolerance induction mechanisms. Since diabetes in humans and NOD mice is thought to be mediated primarily by Th1 cells, regulatory T cells include those cells that are able to downregulate autoreactive Th1 cells. This functional definition implies that regulatory T cells may have various appearances (67).

Deficiencies in certain regulatory T cell subsets, including NKT cells and RT6$^+$ T cells, are associated with diabetes development in humans and animals. As already mentioned BB rats have a general deficiency in peripheral T cells due to the *lyp* mutation. This deficiency especially affects the establishment of the regulatory RT6$^+$ T cell population (39). Another regulatory T cell subset, NKT cells, appears to be functionally deficient in NOD mice, BB rats and humans type 1 diabetes patients (68-72). NKT cells may be the main producers of IL-4 upon initial stimulation (73). It has been postulated that the functional deficiency of NKT cells in the NOD mouse leads to an impairment in Th2 cell function, resulting in a strongly polarized Th1 phenotype (74,75). Indeed, the diabetes-protection induced by transfer of NKT-enriched thymocytes in NOD mice can be neutralised by anti- IL-4 and anti-IL-10 monoclonal antibodies *in vivo*, indicating a role for Th2 cytokines in NKT cell-mediated protection (76). Arguing against a decisive role for the lack of regulatory NKT cells in disease induction are findings in Vα14 TCR transgenic NOD mice. These mice carry a large number of peripheral NKT cells, but are only partially protected against diabetes (75). Thus, a functional defect in NKT cells most likely explains the lack of protection (72).

In conclusion, certain regulatory T cell subsets are (functionally) deficient during diabetes development. Other regulatory T cell subsets may not exert their suppressive actions because of an insufficient stimulation by APC. As already mentioned APC in human diabetes patients, BB rats and NOD mice have maturational defects. Immature APC have been recognized as relatively poor T cell stimulators as compared with mature and activated APC (53,54). Since the stimulation of immunoregulatory T cells requires a more activated APC than the activation of effector T cells, immature APC may be unable to activate regulatory T cells (77). The immature APC present in NOD mice, BB rats and human diabetes patients may thus be unable to activate regulatory T cells and induce peripheral tolerance. Indeed, the stimulation of regulatory T cells as measured in the autologous or syngeneic MLR in both human and animal diabetes is disturbed. Proliferation in the autologous MLR is reduced in at least a subset of diabetes patients in all human studies (51,52,78-85) and to a lesser extent in healthy siblings that are at risk to develop diabetes (51,83). Also in BB rats (40) and NOD mice (48,86), the syngeneic MLR, which is the equivalent of the autologous MLR, is disturbed. The reduced accessory cell function in the MLR in NOD mice has been regarded as a reduced stimulation of regulatory T cells. The BB rat is an excellent model to study putative functional defects of DC in relation to a defective suppressor/regulatory T cell expansion, since this population of T cells specifically expresses specific markers (such as RT6, CD8 and

low levels of CD45RC antigens) (87,88). By using these markers, it has been shown that splenic DC from DP-BB induced expansion of different subsets of regulatory T cells *in vitro* as compared with those from MHC-identical Wistar rats (50). In conclusion, the immaturity of APC present in NOD mice, BB rats and human diabetes patients affects the stimulation of regulatory T cells.

The disturbed autologous or syngeneic MLR can be the result of the already described maturation defects in the DC lineages in diabetes-prone individuals. However, animal studies suggest that also defects in Mϕ function may be involved in the disturbed MLR. In contrast to earlier studies, it has recently been described that the NOD syngeneic T cell proliferation in the MLR is not decreased, but significantly prolonged (63). The prolonged MLR response was observed using isolated splenic DC as stimulator cells, while earlier studies found defective MLR responses using unseparated splenocytes as stimulator cells. This suggests that other splenic cells contribute to the depressed MLR observed in earlier studies. Likely candidates are Mϕ, as the already mentioned high PG production by Mϕ of diabetes-prone individuals leads to a high suppressive activity of Mϕ, inhibiting DC function in the syngeneic MLR.

An insufficient stimulation of regulatory T cells may thus be the consequence of maturational defects in the APC lineages in human and animal diabetes. Studies in the NOD mouse indicate that also altered expression levels of MHC molecules on peripheral APC contribute to the absence of regulatory T cells during diabetes development. We already discussed the unique MHC class II-I-A^{g7} and the implications for central tolerance induction earlier in this review. MHC class II-I-E molecules, or more specifically their absence in NOD mice, appear to be involved in the lack of regulatory T cells that are responsible for the maintenance of peripheral tolerance. NOD mice fail to express MHC I-E molecules due to a deletion of the first exon of the Eα gene (89). The absence of I-E is required for diabetes pathogenesis, since NOD mice transgenic for the wild type I-Eα gene fail to develop diabetes (90,91). Results from transgenic mice with I-E deficiency in specific components of the immune system have indicated that the protection mediated by expression of I-E is not due to negative selection of autoreactive T cells in the thymus (91). Chimeric NOD mice transplanted with a I-E-expressing NOD thymus and regular NOD BM still develop disease, suggesting that the absence of I-E in the thymus of NOD mice is not essential for diabetes development (92). The protection against diabetes in NOD-E mice, that express I-E on both thymus and BM-derived cells, suggests that regulatory cells function normally in the periphery of NOD-E mice (92). The absence of I-E expression on peripheral APC in regular NOD mice may facilitate diabetes development by altering the balance of Th1 versus Th2 cytokines produced by β cell-autoreactive T cells (93). Quantitative differences in I-E expression on peripheral APC that correlate with complete versus partial diabetes resistance in different I-E transgenic lines also correlate with the extent to which these lines activate IL-4-producing T cell responses to GAD65 (93). Although these results obtained for GAD65 should be confirmed using other antigens, it appears that the absence of I-E on NOD peripheral APC may

skew the immune response towards a destructive Th1 phenotype, lacking the balance of regulatory Th2 cells.

In summary, the function of regulatory T cells in the downregulation of autoreactive Th1 cells is disturbed during diabetes development. Some of the regulatory T cells subsets, including $RT6^+$ T cells in BB rats and NKT cells in NOD mice, BB rats and humans, are functionally deficient. Other regulatory T cell subsets are not stimulated because of an immaturity of diabetic APC or because of the absence of I-E expression on peripheral APC.

Conclusion

In both animal and human diabetes, many different aberrations in tolerance induction have been found. Some are specifically occurring in the BB rat, the NOD mouse or humans, others are shared by all (see Tables 1 and 2). In positive selection during central tolerance induction, two major problems can be distinguished. First, a bias exists towards the selection of high-affinity $CD4^+$ T cells, because of poor peptide-binding capacities of diabetes-associated MHC class II haplotypes. Second, these haplotypes are associated with a poor positive selection of regulatory T cells. Thus, the net result of positive selection is a population of high-affinity T cells that is partly deficient in regulatory T cells. Subsequently, disturbances in negative selection contribute to hampered central tolerance induction. First, the structure of the thymic stroma is disturbed, hampering negative selection at the appropriate time point in thymocyte selection. Second, diabetes-associated MHC class II haplotypes do not adequately delete autoreactive $CD4^+$ T cells. Third, antigen processing defects cause an inefficient MHC class I antigen presentation and therefore cause an inadequate deletion of autoreactive $CD8^+$ T cells. Fourth, thymocytes themselves have major defects in apoptosis induction. Negative selection thus ends with a relatively high frequency of autoreactive T cells. Taken together, a T cell repertoire enriched for autoreactive high-affinity T cells is released into the periphery after the inadequate central tolerance induction.

Defective peripheral tolerance mechanisms provide additional problems for the maintenance of self-tolerance. The impaired APC-mediated T cell stimulation in conjunction with the constitutive APC PGS_2 expression hamper AICD, whereas also TCR signaling and apoptosis defects appear to be involved in the suboptimal function of this peripheral tolerance mechanism. Autoreactive T cells, once they have been activated, survive severe IL-2 deprivation and thus bypass cytokine withdrawal death. Finally, the suppressive actions of regulatory T cells are affected by the absence of certain regulatory T cell subsets and the failure of APC to stimulate those regulatory T cells that are present. Thus, the high-affinity autoreactive T cells that reached the periphery do not die upon activation because of a disturbed AICD and a resistance to cytokine deprivation and will not be suppressed in their functions because of an absence of regulatory T cells. The obvious end result of this defective tolerance induction is the development of autoimmunity.

In this review, we have summarized many problems that T cells encounter during their education in diabetes-prone humans and animals. Knowing that stromal cells in the thymus act as teachers and developing thymocytes as students, we could state that underqualified teachers try to teach unresponsive students about what these students should or should not do. This obviously disturbed T cell education underlies the development of the T cell-mediated autoimmune disease diabetes in humans, BB rats and NOD mice.

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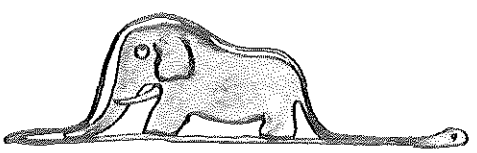
Chapter 1.3

Aim and outline of this thesis



Aim and outline of this thesis

In the previous chapters, it has become clear that type 1 diabetes in humans and its animal models are multifactorial diseases, in which several genes and environmental factors are involved. In Chapter 1.1, the pathophysiology leading to diabetes in humans and animals has been reviewed. The exact etiology of the disease, however, remains unclear. In Chapter 1.2, several immune defects have been described that predispose NOD mice, BB rats and humans to the development of autoimmunity. The immune defects in diabetes-prone individuals, however, are not specific for diabetes. Many of these defects can also be found in other autoimmune diseases. Thus, defects only in the immune system can not explain why the β cells in the pancreatic islets of Langerhans are the main target of the autoimmune reaction during diabetes development, whereas most other organs are spared. In fact, the presence of islet-reactive T cells is not unique to NOD mice; such cells have also been demonstrated in non-autoimmune prone mouse strains. Thus, the lack of immunological tolerance cannot be the only cause of diabetes.

In this thesis, we approach the hypothesis that abnormalities in the function and/or phenotype of the β cells in the islets of Langerhans render them susceptible for the autoimmune attack. Thus, according to this hypothesis, the target organ itself determines the organ-specificity of the autoimmune disease. We follow a two-hit-model: in addition to a susceptibility in the immune system that predisposes to the loss of tolerance in general, a susceptibility of the target organ is needed for organ-specific autoimmunity. In this study, we are especially interested in the interaction between target organ and non-lymphoid cells, since the latter have been shown to play a role in both the immune and the endocrine system. Moreover, they are the first cells to appear in the inflammatory lesion. The NOD mouse was chosen for these studies, since the pathogenic process in this mouse bears many resemblances to the pathogenesis of human type 1 diabetes.

We first studied the identity of subtypes of non-lymphoid cells in pancreatic inflammatory infiltrates in NOD mice in which kinetically different pathogenic processes occur (Chapter 2.1). In this study, we correlated the presence of specific subtypes of myeloid cells with aggressiveness of the lesion. We next searched for abnormalities in the function and phenotype of the islets, which could potentially trigger the initial infiltration with non-lymphoid cells. We found that NOD mice have an enhanced percentage of abnormally large islets, designated 'mega-islets' (Chapter 3.1). Importantly, the first infiltrating inflammatory APC are preferentially associated with these mega-islets. Moreover, we determined that the formation of these mega-islets, with concomitant infiltration, is especially pronounced in diabetes-prone NOD females and orchidectomized NOD males as compared to diabetes-resistant NOD males (Chapter 3.2). Thus, the kinetics of formation of mega-islets correlate with the development of disease.

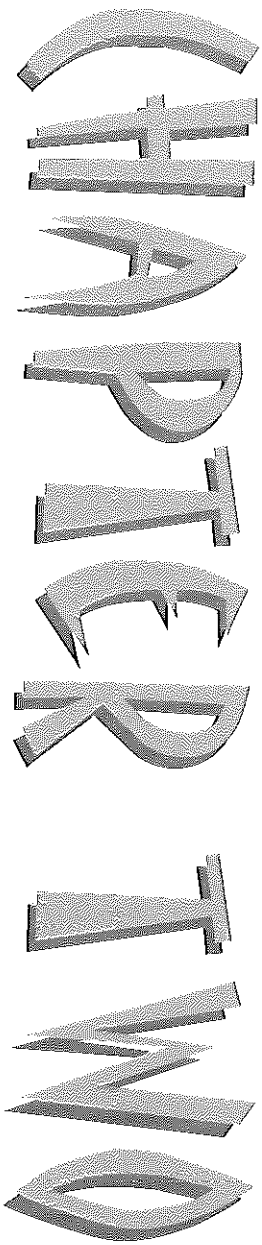
In search of the possible causes of mega-islet formation, a hyperinsulinemia was found that preceded islet hypertrophy. Islet hypertrophy could thus be the consequence of an

increased demand on the β cells that could not be fulfilled by the available numbers. The hyperinsulinemia, in turn, may be related to metabolic abnormalities that NOD mice experience during their develoment in utero (Chapter 3.3). However, also infiltrating cells may contribute to the hyperinsulinemia, since this correlates in time with the start of islet infiltration with APC. Cultures of isolated islets of Langerhans and isolated DC showed that infiltrating DC are indeed able to influence β cell activity (Chapter 3.4). Furthermore, the hyperinsulinemia may also partly be triggered by a hyperglucagonemia (Chapter 3.5). The association of hyperinsulinemia with hyperglucagonemia suggests a generic abnormality in islet function. This is possibly related to ontogenetic deviation, since we find evidence for an abnormality in islet development in NOD mice (Chapter 3.5).

The presence of β cell abnormalities in neonatal NOD mice may suggest that these early islet abnormalities are involved in the triggering of the organ-specific autoimmunity. However, although earlier reports assume that autoimmune infiltration starts after weaning (i.e. at 3 weeks of age), an early influx of inflammatory cells can already be recognized in neonatal mice (Chapter 3.6).

In conclusion, we found evidence for several abnormalities in the islets of Langerhans that potentially trigger the organ-specificity of diabetes in the NOD mouse. Taking these findings into account, we discuss the putative etiology of NOD diabetes and present a hypothetical scenario for the pathogenesis of the disease (Chapter 4.1). Furthermore, we discuss the implications of these findings for human type 1 diabetes.

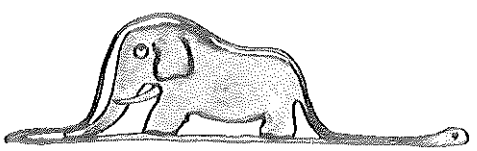
Non-lymphoid cells in NOD mouse insulitis



2.1 Subsets of macrophages and dendritic cells in nonobese diabetic mouse pancreatic infiltrates: correlation with the development of diabetes
Lab Invest 80(1):23-30 (2000)

Chapter 2.1

Subsets of macrophages and dendritic cells in NOD mouse pancreatic inflammatory infiltrates: correlation with the development of diabetes



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Summary

Islet-specific T cells are essential in the development of autoimune diabetes. The role of non-lymphoid cells is relatively unclear, although infiltration of dendritic cells and macrophages is the first sign of islet autoimmunity in diabetes-prone nonobese diabetic (NOD) mice. BDC2.5 is one of the autoreactive T cell clones isolated from NOD mice. Transfer of BDC2.5 T cells into young NOD mice accelerates diabetes development, whereas transgenic expression of the BDC2.5 T cell receptor on NOD T cells (BDC2.5 TCR-Tg NOD) markedly reduces diabetes development.

We show that, although the same antigen-specificity is involved, both models differ significantly in insulitis. BDC2.5 TCR-Tg NOD mice develop an extensive, but non-aggressive, peri-insulitis by 3 weeks of age. In these large peri-islet infiltrates, resembling secondary lymphoid tissue, $BM8^+$ macrophages (Mϕ) are virtually absent. In contrast, BDC2.5 T cell clone transfer results in an aggressive insulitis with small infiltrates, but relatively large numbers of $BM8^+$ Mϕ. Infiltration of $BM8^+$ Mϕ therefore correlates with islet destruction. This is, however, not observed for all Mϕ; Monts-4^+ Mϕ follow a reverse pattern and are present in higher numbers in BDC2.5 TCR-Tg than in transferred mice. ER-$MP23^+$ Mϕ are reduced in both transferred and transgenic mice compared with wild-type NOD.

Thus, this study underlines and extends previous data suggesting that Mϕ are implicated in both early and late phases in diabetes development. Furthermore, our data imply that subsets of non-lymphoid cells have different roles in diabetes development. It is, therefore, important to recognize this heterogeneity when interpreting both in vivo and in vitro studies concerning non-lymphoid cells in diabetes.

Introduction

Type 1 (insulin-dependent) diabetes mellitus (type 1 diabetes) is the result of an autoimmune destruction of the β cells in the islets of Langerhans (1). The disease is characterized by a very long pre-clinical period. In this period, several types of leukocytes accumulate first around the islets (a process called peri-insulitis) and subsequently infiltrate into the islets (insulitis). It is only when the vast majority of pancreatic β cells are destroyed that the alteration of glucose-linked metabolism generates the classical signs of diabetes. The extensive pre-clinical period provides a tool for prediction and prevention of the disease.

To study the development of type 1 diabetes, animal models such as the nonobese diabetic (NOD) mouse and the BioBreeding (BB) rat have been employed. In both models, the first sign of islet autoimmunity is an accumulation of antigen-presenting cells (APC) around and in the islets (2,3). These APC include various types of dendritic cells (DC) and macrophages (Mϕ). DC and Mϕ are characterized in the NOD mouse by markers such as CD11c, ER-MP23 and MOMA-1 (3) and in the BB rat by MHC class II and ED1, but absence of acid phosphatase (2). The exact role of the different subtypes of myeloid cells is not known. Early infiltrating DC and accessory Mϕ are thought to pick up relevant autoantigens and to migrate to the draining lymph nodes to present the islet antigens in the paracortical area to recirculating lymphocytes. B cells (4) and $CD8^+$ T cells (5) are probably also important in these very early phases of the insulitis process, but these cells occur only at relatively low frequencies. In time, the phase of peri-islet accumulation of APC is followed by a reaction of the draining lymph nodes and an accumulation of B and T lymphocytes around the islets, the latter in close apposition to the already accumulated APC (3). The actual β cell killing is histologically characterized by an infiltration into the islets of Mϕ, whereas $CD8^+$ T cells (5) are also seen to infiltrate the islet. The histopathological picture of infiltration with scavenger type Mϕ is compatible with the view that β cell killing is at least in part a Th1 phenomenon (6,7), where T cell-derived cytokines such as interferon-γ activate Mϕ.

In the destruction of the β cells, T cells are crucial. From the NOD mouse, several islet-specific diabetogenic T cell clones have been isolated. One of these is the $CD4^+$ T cell clone BDC2.5 (8), reactive to an as-yet unknown antigen localized on the membrane of the β cell granule (9). Its in vitro cytokine profile is consistent with the phenotype of a Th1 cell (10) and the clone is able to transfer insulitis and diabetes to young NOD mice (11). Although the transferred insulitis is characterized by a relatively small lymphocyte infiltration, a rapid progression to diabetes (within 2 to 3 weeks) is observed (11).

Furthermore, a mouse model has been developed based on the transgenic (Tg) expression of the rearranged T cell receptor (TCR) genes from the BDC2.5 T cell clone on a NOD background (12). In contrast to the wild-type NOD, this TCR-transgenic NOD model is characterized by an extensive lymphocyte infiltration from 3 weeks of age onwards. However, the

diabetes incidence of BDC2.5 TCR-Tg mice on the full NOD genetic background is strongly reduced, compared with wild-type NOD mice.

Despite the use of the same diabetogenic TCR in both BDC2.5 models, different kinetics of disease development are observed in these mice. We asked whether these kinetic differences could be related to differences in the presence of non-lymphoid cell subtypes, and thus if these models could provide us with the opportunity to study the role of subtypes of non-lymphoid cells in diabetes development. Therefore, we have analyzed the pancreatic infiltration of various subsets of DC ($CD11c^+$, NLDC-145^+), Mϕ ($BM8^+$, Monts-4^+, ER-$MP23^+$), T cells ($CD4^+$, $CD8^+$) and B cells ($B220^+$) in both the BDC2.5 T cell clone transfer and the BDC2.5 TCR-transgenic (BDC2.5 TCR-Tg) NOD model.

Results

Pancreatic histopathology of BDC2.5 TCR-Tg NOD mice as compared with wild-type NOD mice

The sequence of histopathological events preceding diabetes in wild type NOD mice is described in Table 1.

Table 1: Insulitis in wild-type NOD mice.

stage 0:	Intact islet as observed in non-diabetes prone mice
stage I (wk 3):	Intact islet, but an increase in perivascular and periductular extracellular matrix and a perivascular increase of $CD11c^+$, ER-$MP23^+$, MOMA-1^+ and $BM8^+$ Mϕ and DC
stage II (wk 4-7):	Islet surrounded by DC and Mϕ with above-mentioned phenotypes; infiltration of islets by $CD11c^+$ DC; a major infiltration by lymphocytes is still absent
stage III (wk 7-10):	Parainsular recruitment of substantial numbers of $CD4^+$ and $CD8^+$ T lymphocytes and some B lymphocytes to the accumulation of DC and Mϕ
stage IV (week 10-17):	Lymphocytes surround the islet (peri-insulitis)
stage V (> wk 17):	Lymphocytes and ER-$MP23^+$ and $BM8^+$ Mϕ infiltrate the islet
stage VI (>> 17 wk):	Endstage; no insulin-producing cells detectable

A representative infiltrate in a female NOD mouse is shown in Figure 1. In wild type NOD mice, $CD11c^+$ DC and ER-$MP23^+$ Mϕ were the first inflammatory cells appearing at the circumference of the islets at 4 to 7 weeks (3). The influx of these cells was substantial in the early phases, whereas only a few T cells (if any) were present at this stage. Thereafter, a slowly increasing recruitment of T cells and of APC was observed.

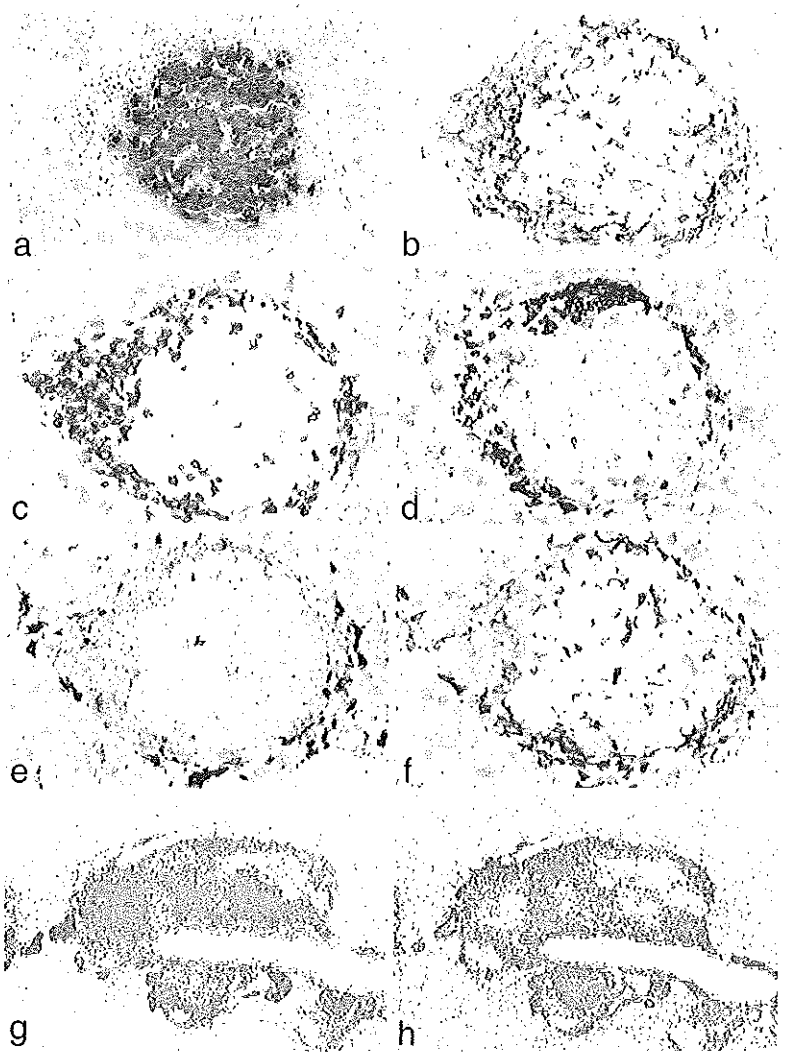

Figure 1: Immunohistological characterization of a representative insulitis (a to f; original magnification, x200) and of a relatively rare lymphoid tissue-type of peri-insulitis (g and h; original magnification, x45) in a female 15-week-old NOD mouse. (a) Anti-insulin staining, showing the β cell mass; (b) CD11c, showing peri- and intra-islet accumulation of these DC; (c and d) CD3 (T cells) and B220 (B cells), respectively. Note that lymphocytes initially accumulate around the islet (peri-insulitis); e) ER-MP23, showing that histiocytes accumulate at the islet-edge; (f) BM8, showing a peri-and intra-islet accumulation of these Mφ; (g and h) CD3 (T cells) and B220 (B cells), respectively. Note the high grade of organization as normally found in secondary lymphoid tissues and in the BDC2.5 TCR-Tg NOD mouse.

In the BDC2.5 TCR-Tg NOD, however, both APC and lymphocytes were recruited at the same time. From about 3 weeks of age onwards, both APC and lymphocytes were present in substantial numbers at the edge of the islets, and large peri-insulitis areas were evident.

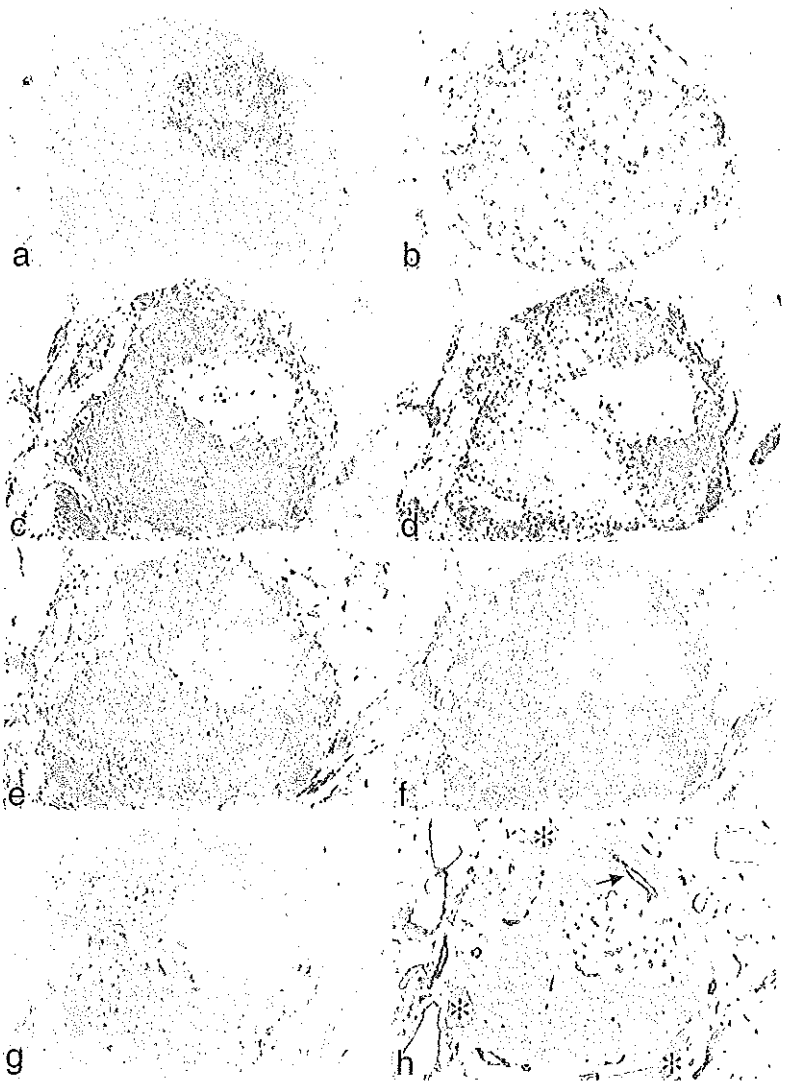

Figure 2: Immunohistological characterization of a representative peri-islet lymphoid cell accumulation in a BDC2.5 TCR-Tg female NOD mouse, 4 weeks old. Original magnification x90. (a) Anti-insulin staining, showing the position of the β cell mass; (b) CD11c, showing the accumulation of DC at the islet-edge and in between the accumulated lymphocytes; (c and d) CD3 (T cells) and B220 (B cells) respectively, showing the high grade of organization of the accumulated peri-islet lymphocytes in separate T- and B cell areas reminiscent of secondary lymphoid tissue; (e) ERMP-23, showing that histiocytes, one of the first types of Mϕ accumulating at the islet-edge in wild-type NOD insulitis, can be found only in small numbers at the periphery of the secondary lymphoid tissue; (f) BM8, showing the conspicuous absence of scavenger type Mϕ, similar to the situation seen in secondary lymphoid tissues; (g) Monts-4, showing that the lymphocyte peri-islet accumulation contains Monts-4$^+$ Mϕ normally seen in secondary lymphoid tissues; (h) ER-MP12, showing endothelial cells, particularly HEV (arrow) and lymphatics (asterisk) characteristic of the secondary lymphoid tissue nature of the peri-islet lymphoid cell accumulations.

Moreover, the number of ER-MP23$^+$ Mϕ was either strongly decreased or they were absent from the islets (Fig. 2e). In the phase of peri-insulitis, we observed again clear differences between the BDC2.5 TCR-Tg NOD and the wild-type NOD. From 3 weeks of age, the large peri-insular infiltrates stayed very pronounced in the BDC2.5 TCR-Tg NOD, until the end of our observation time. In wild-type NOD, however, well-developed peri-insular infiltrates containing substantial numbers of lymphocytes could not be seen before the age of 9 weeks (3). In the BDC2.5 TCR-Tg NOD, massive lymphocyte infiltrates were clearly larger than the ones found in the wild-type NOD (Fig. 2), although the islet itself was not affected. The large lymphocyte infiltrates found in the transgenic animals had a high degree of organization. B and T lymphocytes were localized in separate areas (Fig. 2, c and d) within the infiltrate. As expected in an MHC class II restricted TCR transgenic mouse, the T cell infiltrate consisted primarily of CD4$^+$ T cells (results not shown). DC were found in all compartments of the inflammatory infiltrate (Fig. 2b), but especially at the interface of intact β cells and the T cell infiltrate (Fig. 2b). Moreover, two DC populations, characterized by CD11c and NLDC-145, were only partially overlapping. CD11c$^+$ DC were seen accumulated at the border of the T cell infiltrate and intact β cells, and were hardly present in the T cell area or in the islet mass (fig. 2b). NLDC-145$^+$ DC were also found at the β cell/T cell border, but - in contrast to CD11c$^+$ DC - were also present intermingled with T cells and in the islet mass (13). In addition, high endothelial venules (HEV) as well as lymph vessels could be found in the T lymphocyte peri-insular areas (Fig. 2h). The peri-insular infiltrates of the BDC2.5 TCR-Tg NOD mouse thus histologically resemble secondary lymphoid tissue, especially mucosa-associated lymphoid tissue (eg, Peyer's patches). The finding of Monts-4$^+$ Mϕ, normally present mainly in lymphatic tissue (14), is in agreement with this (Fig. 2g). Occasionally, this lymphatic tissue adjacent to islets was also found in advanced stages in wild-type NOD mice (Fig. 1, g and h), although the majority of NOD islets had a peri-insulitis with a non-characteristic architecture. Infiltration of leukocytes into the islets themselves was hardly observed in BDC2.5 TCR-Tg mice.

In wild-type NOD mice, destructive insulitis was histologically characterized by an early infiltration of BM8$^+$ Mϕ into the islets starting at 7 to 10 weeks. This infiltration of BM8$^+$ Mϕ into the islets was especially pronounced in diabetes-prone female NOD mice, and was considerably less in male NOD mice that show a lower diabetes incidence. Destructive insulitis involving the influx of lymphocytes into the islet mass occurred from 15 weeks onwards. In contrast to the regular NOD insulitis, BM8$^+$ Mϕ were virtually absent in the BDC2.5 TCR transgenic NOD infiltrates, especially in later stages of infiltration (Fig. 2f).

In summary, BDC2.5 TCR-Tg NOD mice developed extensive infiltrates resembling secondary lymphoid tissue, with a virtual absence of BM8$^+$ Mϕ. Although the infiltrates developed early and were extensive, they did not lead to β cell destruction.

Pancreatic histopathology of BDC2.5 T cell clone transfer as compared with wild-type NOD mice

In young (14 days) NOD mice receiving transfers of the BDC2.5 T cell clone, a very fast and aggressive insulitis was observed. Almost no insulin-producing cells could be detected at 10 days after the transfer. In relation to this, no sequential accumulation of APC and lymphocytes was observed. Only a very limited accumulation of ER-MP23+ Mϕ, which in wild-type NOD are among the first to surround the islets, was found (Fig. 3e). In contrast, there was an increased accumulation of $CD11c^+$ DC, simultaneously with an accumulation of lymphocytes (Fig. 3b).

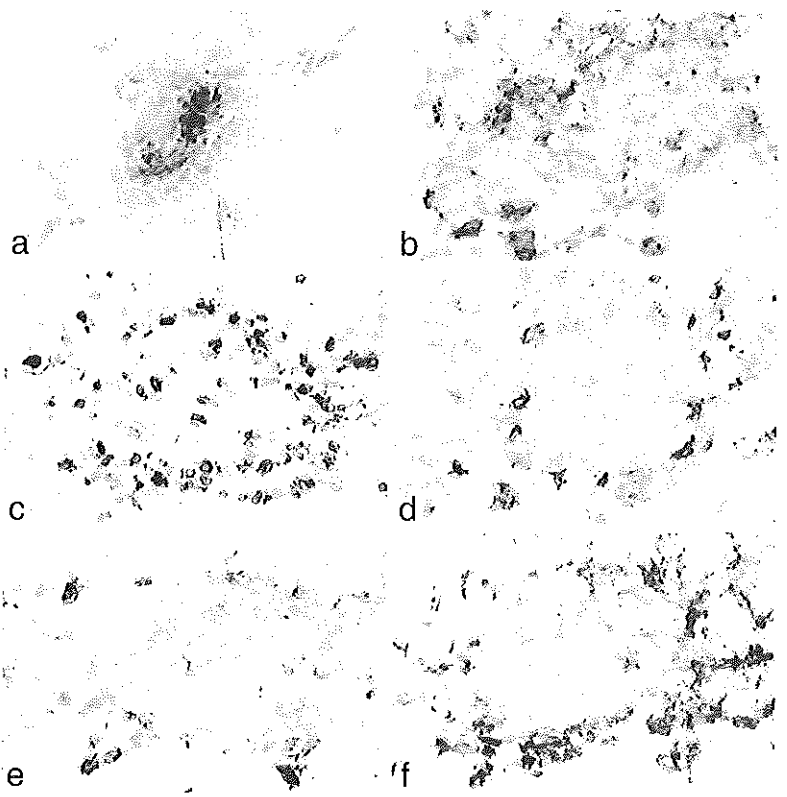

Figure 3: Immunohistological characterization of a representative insulitis in a female NOD mouse (3 weeks old) transferred with the BDC2.5 T cell clone (10 days after the first transfer). Original magnification x360. (a) Anti-insulin staining, showing the β cell mass; (b) CD11c, showing a relatively enhanced accumulation of DC at the islet edge as compared with wild-type NOD insulitis; (c and d) CD3 (T cells) and B220 (B cells) respectively. Note that there is hardly any peri-insulitis, but a relatively small infiltration of single cells as compared with the wild-type NOD and certainly as compared with the BDC2.5 TCR-Tg NOD; (e) ER-MP23, showing that histiocytes accumulate at the islet-edge like in the wild-type NOD insulitis; (f) BM8, showing an enhanced peri-and intra-islet accumulation of these Mϕ.

Compared with wild-type NOD mice and certainly to BDC2.5 TCR-Tg mice, the accumulation of lymphocytes near the islets was minor. In the exocrine pancreas, few B and T cells were found. B cells were especially present around the islets, and a few were also infiltrating the islets (Fig. 3d). T cells (Fig. 3c) consisted of both $CD4^+$ and $CD8^+$ cells; using immunofluorescent staining on isolated peri-islet T cells it has previously been determined that $CD4^+$ T cells appeared in larger numbers as compared with $CD8^+$ T cells (K. Haskins, personal communication). Infiltrates in BDC2.5 T cell clone transferred mice did not show any degree of histological organization. In addition, only limited numbers of Monts-4^+ Mϕ were found (not shown).

The most conspicuous observation in the BDC2.5 T cell clone-transferred mice was the strong and early infiltration with $BM8^+$ scavenger type Mϕ around the islets and into the islet mass (Fig. 3f). $BM8^+$ Mϕ were also seen scattered throughout the exocrine pancreas. This highly increased involvement of $BM8^+$ Mϕ contrasted the almost complete absence of such Mϕ in the BDC2.5 TCR-Tg NOD inflammatory infiltrates. Also in the wild-type NOD insulitis, $BM8^+$ Mϕ although infiltrating the islets, were found in significantly lower numbers compared with the transfer model, especially in male but also in female mice.

In summary, BDC2.5 T cell clone-transferred NOD mice developed small unorganized infiltrates, with a high number of $BM8^+$ Mϕ. These infiltrates appeared to be highly aggressive, as evidenced by the rapid loss of β cells.

Discussion

Our data show that the (peri-)insulitis in the BDC2.5 T cell clone transfer model, the BDC2.5 TCR-Tg NOD model, and the wild type NOD model follow distinct immunohistomorphological patterns with different involvement of non-lymphoid cell types. In the transfer model, a rapid destruction of the β cells is associated with a progressive but relatively mild lymphocyte infiltration, whereas compared with wild-type NOD there is an extensive infiltration with $BM8^+$ Mϕ. In contrast, the BDC2.5 TCR-Tg NOD model showed a very early development and a long-lasting presence of lymphoid tissue around otherwise intact islets, although there was a low incidence of diabetes. This lymphoid tissue was characterized by T and B cell zones, specific Monts-4^+ lymph nodal Mϕ and $CD11c^+$ DC, the presence of HEV, but a conspicuous absence of $BM8^+$ Mϕ.

The BDC2.5 T cell clone transfer model and the BDC2.5 TCR-Tg model also shared some histopathological features that differed from the wild-type NOD. The wild type NOD showed an initial phase of peri-islet accumulation of DC and Mϕ but a virtual absence of lymphocyte infiltration. However, both BDC2.5 models showed a simultaneous early peri-islet accumulation of APC and lymphocytes.

These data reveal several interesting differences in the kinetics of insulitis in NOD mouse models. In the TCR-Tg and in the transfer model, there is no separate phase of APC

infiltration preceding lymphocyte infiltration, whereas this phase is present in wild-type NOD. The absence of a separate phase of APC accumulation indicates that the influx of lymphocytes takes place with accelerated kinetics in both BDC2.5 models. This acceleration of infiltration is most likely due to an increased frequency of autoreactive T cells in both BDC2.5 models compared with wild-type NOD. Because of the relatively low frequency of islet-specific T cells in the initial naïve T cell pool of the wild-type NOD, its islet antigen-loaded DC need to interact with many naïve T cells to encounter an islet antigen-specific-TCR. Such conditions are initially most optimally met in the pancreas-draining lymph nodes because of the recirculation of T cells via lymph nodal HEV and a specific accumulation in these nodes of islet antigen-loaded DC via the afferent lymph. The likelihood of such an encounter is minimal in the early small T cell infiltrates around the wild-type NOD islet. In both the BDC2.5 TCR-Tg and T cell clone transfer model, there are already many islet-specific T cells in the repertoire early in life. This increases the likelihood of a specific DC-T cell encounter at the islet-edge itself. Therefore, a presentation of autoantigens in lymph nodes may not be required in both BDC2.5 models, whereas it is obligatory in wild-type NOD for clonal expansion of T cells.

Accelerated infiltration, however, does not lead to β cell destruction in the BDC2.5 TCR-Tg NOD. The large, non-destructive infiltrates observed in this model are also observed in other TCR-Tg mouse-models with the cognate antigen expressed under the insulin promoter or only locally expressed in the thyroid (15). Autoreactive T cells are not deleted in the thymus in these TCR-Tg mice because of a lack of systemic expression of the autoantigen. The large peri-insular infiltrates developing in such models also do not lead to actual destruction, since the autoreactive T cells present likely have an impaired function. This impaired function of autoreactive T cells is reflected in a change in the sensitivity to TCR stimulation leading to diminished in vitro responsiveness to antigen and poor in vivo ability to provide B cell help (15). It remains to be determined whether the T cells in the BDC2.5 TCR Tg NOD are similarly dysfunctional. An alternative and, in our view, a more likely explanation for the strongly reduced β cell destruction in BDC2.5 TCR-Tg NOD may be found in the number of $BM8^+$ Mϕ in the inflammatory infiltrate. These $BM8^+$ Mϕ are virtually absent in BDC2.5 TCR-Tg NOD, whereas they are abundantly present in the transfer model. In the wild-type NOD, $BM8^+$ Mϕ infiltrate the islets in diabetes-prone females, but not in diabetes- (but not insulitis-) resistant males (3). Together, these data strongly suggest that $BM8^+$ Mϕ are a histological marker of the progression from peri-insulitis to β cell destruction and diabetes.

Various roles for these $BM8^+$ Mϕ could be envisaged. They may play a role simply in removing the cellular debris resulting from the autoimmune inflammation. Alternatively, $BM8^+$ Mϕ could be the ultimate effectors in the β cell killing, possibly stimulated by T cells. It is also possible that $BM8^+$ Mϕ induce some minor β cell damage that - by virtue of enhanced antigen release - leads to antigenic spreading and a further local triggering of T cells. In that case, the peri-insular accumulated APC have a role in a final local T cell stimulation of the infiltrated T cells. Such a mechanism is compatible with a two-step model for

the development of autoimmunity (16). In the first step, initial priming of islet-specific T cells with antigen occurs in the lymph node and results in the accumulation of lymphocytes in the target organ. In the second step, restimulation in the target organ by tissue-localized APC causes the lymphocytes to become destructive and clinically evident autoimmunity occurs. In general, the necessity of local restimulation of T cells for their final activation would protect other tissues from bystander-induced damage by T cells that are accidentally activated in other organs. The local microenvironment would then be an important determinant in APC function and thus in the ultimate outcome of the autoimmune infiltration.

Which factors could determine the differential influx of $BM8^+$ Mϕ in transgenic and transfer models? An important determinant may be an extremely high frequency of unactivated BDC2.5 TCR-carrying T cells in the BDC2.5 TCR-Tg versus a mildly raised frequency of activated and mature BDC2.5 T cells in the transferred NOD mice. Also in other models, the frequency of autoreactive T cells has been shown to determine the fate of these cells (17). Another determinant may be an almost complete absence of $CD8^+$ T cells, due to the MHC class II restricted TCR in the BDC2.5 TCR-Tg NOD mice compared with BDC2.5 T cell clone-transferred and wild-type NOD mice. However, transfer of CD8+ T cells in BDC2.5 TCR-Tg mice did not accelerate disease (18), making this possibility less likely. Alternatively, in the BDC2.5 TCR-Tg NOD mice, regulatory interactions between the transgenic T cells and B cells, or $CD4^+$ T cells expressing endogenous TCRα chains, may serve to attenuate the activity of the transgenic T cells. This possibility is likely given that, if bred on a NOD*scid* background, all of the BDC2.5 TCR-Tg mice develop diabetes by 3 to 5 weeks of age (19). In the BDC2.5 TCR-Tg NOD*scid*, there is a very extensive early infiltration, which is rapidly followed by full-blown diabetes. The infiltrate resembles pancreatitis and, in contrast to those observed in the BDC2.5 TCR-Tg NOD, is highly disorganized and invasive. The infiltrate comprises many $CD4^+V\beta4^+$ T cells, likely expressing the cognate BDC2.5 TCR, and $BM8^+$ Mϕ with a few ER-$MP23^+$ Mϕ scattered throughout.

On the basis of the differential involvement of non-lymphoid cell subsets in the presently investigated BDC-2.5-TCR-based models, we suggest that subtypes of non-lymphoid cells play different roles in diabetes development. Clearly, this notion needs further experimental investigation to delineate the functions of the distinct subtypes. However, this heterogeneity should be recognized when interpreting in vivo and in vitro studies concerning the role of non-lymphoid cells in diabetes development.

Materials and methods

Animals

BDC2.5 TCR-Tg NOD mice and NOD mice used for T cell clone transfer studies were bred and maintained in the animal facilities at the Barbara Davis Center and the School of

Medicine at the University of Colorado Health Sciences Center in Denver, Colorado. The incidence of diabetes in NOD/bdc mice is 50% to 60% in females and 30% to 40% in males by 28 weeks of age. In the Denver colony, the BDC2.5 TCR-Tg NOD mouse incidence of diabetes is 10% to 15% for both female and male mice by 28 weeks of age, and is thus strongly reduced compared with wild-type NOD mice. This was not the case with earlier backcrosses of BDC2.5 TCR-Tg, which only showed an extended lag period between initiation of insulitis and onset of diabetes (12). We wish to emphasize that the mice in the present study were BDC2.5 TCR-Tg on a full NOD background

BDC2.5 TCR-Tg NOD mice were killed at 3 to 4, 6 to 7, 12, or 16 weeks of age. For clone transfers, mice (12 to 13 days of age) received an injection of 10^7 T cells, ip, and a week later received a second injection, again 10^7 cells. Animals were killed 3 days after the second injection. The cloning and maintenance of the BDC-2.5 T cell clone was previously described (8). Wild-type NOD mice were bred and kept at the facility of the Department of Immunology, Erasmus University in Rotterdam, The Netherlands. The incidence of diabetes in this colony is 80% to 90% in females and 50% to 60% in males by 30 weeks of age.

Immunohistochemistry

For each age, 5 mice were killed by cervical dislocation, pancreases were removed, embedded in OCT compound and frozen on dry ice. Tissues were stored at -80°C until immunohistochemistry was performed. Before sectioning, microscope slides were coated with a solution of 0.1% gelatin/0.01% chromium-alum. Pancreas cryostat sections of 6 µm were prepared, coded and fixed for 2 min in 2% pararosaniline (20). After a wash with phosphate buffered saline with 0.1% Tween-20 (Merck-Schuchardt, Hohenbrunn bei München, Germany) (PBS/Tween), slides were incubated with first step antibodies (Table 2) for 30 min at room temperature. BM8 (BMA Biomedicals, Augst, Switzerland) was used diluted 1:30 in PBS/Tween, anti-insulin (DAKO, Carpinteria, California) was used diluted 1:100 in PBS/Tween. All others were supernatants from hybridomas cultured at our department and used undiluted. Subsequently, slides were washed with PBS/Tween and incubated with peroxidase-conjugated goat-α-hamster-immunoglobulins (Ig) (to detect N418), rabbit-α-guinea-pig-Ig (to detect α-insulin) or rabbit-α-rat-Ig (to detect all others) second step antibodies in the presence of 2% normal mouse serum for 30 min at room temperature. After an additional wash with PBS/Tween, slides were incubated with 0.05% (w/v) Ni-di-amino-benzidine (Ni-DAB) with 0.02% H_2O_2 and washed in water after 3 min. Finally, slides were counterstained for 3 min in nuclear fast red, dehydrated in a graded ethanol series, and mounted. For each staining run, one slide was stained with second antibody only as a control for endogenous peroxidase activity and non-specific binding of the second step, and a section of spleen was included as a positive control.

Table 2: Antibodies used for immunohistochemical study (21)

	Specificity	Source
Monoclonal Ab		
RA3.6B2	CD45R-antigen; B220-antigen	R. Coffman
BM8	Mϕ with phagocytosing capacity	BMA
ER-MP12	CD31-antigen; hematopoietic progenitors, endothelia	P. Leenen
ER-MP23	mouse Mϕ galactose-/N-acetyl galactosamine specific C-type lectin (MMGL); connective tissue Mϕ	P. Leenen
KT3	CD3-antigen	ATCC
53-6.72	CD8-antigen; Lyt2	ATCC
Monts-4	Mϕ in lymphoid areas	M. Jutila
H129-19	CD4-antigen; MT4	P. Naquet
N418	CD11c-antigen; DC	ATCC
NLDC-145	DEC205 ; DC	G. Kraal
Polyclonal Ab		
α-insulin	insulin	DAKO

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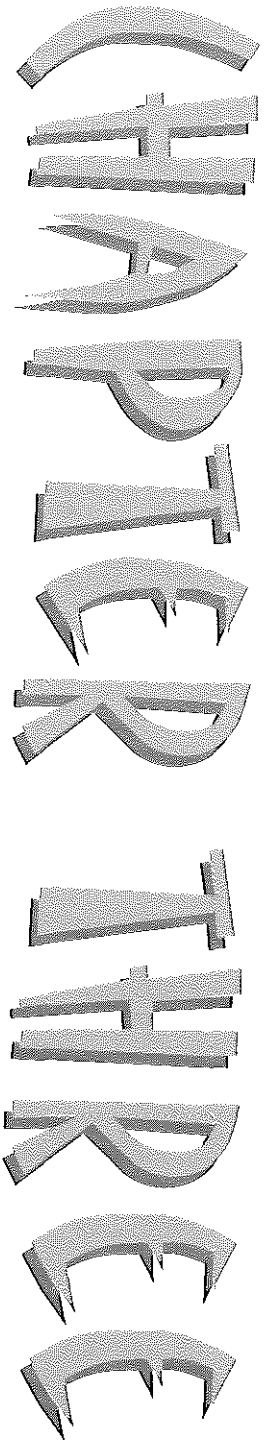


Prediabetic NOD islet abnormalities in relation to insulitis

3.1 Islet abnormalities associated with early influx of dendritic cells and macrophages in NOD and NODscid mice.
Lab Invest 80(5):in press (2000)

3.2 Sex steroids influence pancreatic islet hypertrophy and subsequent autoimmune infiltration in NOD and NOD*scid* mice.
Submitted

3.3 Increased β cell activity in neonatal nonobese diabetic mice: *in situ* hybridization analysis of preproinsulin transcriptional levels.
Submitted

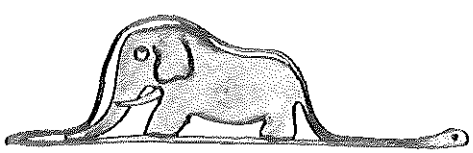
3.4 NOD mouse dendritic cells aberrantly stimulate islet insulin release in vitro.
Submitted

3.5 Islet α cell disturbances from birth onwards in mice with the nonobese diabetic genetic background.
Submitted

3.6 Neonatal pancreatic infiltration by dendritic cells and macrophages in mice with nonobese diabetic (NOD) or non-autoimmune genetic background.
Submitted

Chapter 3.1

Islet abnormalities associated with an early influx of dendritic cells and macrophages in NOD and NOD*scid* mice



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Summary

In the nonobese diabetic (NOD) mouse model for type 1 diabetes mellitus, the inflammatory infiltration of islets starts with an influx of dendritic cells (DC) and macrophages (Mϕ) at about 4 weeks of age. Around this time, NOD mice show endocrine abnormalities, indicated by a transient hyperinsulinemia that lasts until 8 weeks of age. Subsequently, they develop abnormally large islets of Langerhans, here designated as "mega-islets". NOD*scid* mice, which lack functional lymphocytes, also exhibit transient hyperinsulinemia, but to a lesser extent.

First, to determine the role of lymphocytes in the morphological islet abnormalities, we compared 6-week-old (prediabetic) NOD and NOD*scid* females regarding mega-islet development and accumulation of antigen-presenting cells (APC), particularly $CD11c^+$ DC and $ERMP23^+$ Mϕ. In NOD*scid* mice, early APC infiltration and mega-islets were present, but less marked compared to NOD mice, thus suggesting a role of lymphocytes in mega-islet formation. In both NOD and NOD*scid* mice, the APC infiltration was predominantly found around the mega-islets, suggesting a relationship between both parameters. Second, to analyze the role of β cell hyperactivity in mega-islet formation, we studied the effect of short-term prophylactic insulin treatment on these parameters. Prophylactic insulin treatment decreased the percentages of mega-islets in both NOD and NOD*scid* mice, indicating that β cell hyperactivity is also involved in mega-islet formation.

In conclusion, mega-islet formation in mice with the NOD genetic background takes place under influence of both β cell hyperactivity and leukocytes.

Introduction

Type 1 (insulin-dependent) diabetes mellitus (type 1 diabetes) is a T cell mediated autoimmune disease, in which the insulin-producing cells in the islets of Langerhans are attacked and destroyed by infiltrating leukocytes (1-3). The disease is characterized by a long pre-clinical period: symptoms of diabetes only develop when less than 10% of the β cell mass is left. During this pre-clinical period, various autoantibodies directed against β cell antigens, including insulin and GAD (4-6), are circulating. This pre-clinical period provides an opportunity for prediction and prevention, and therefore needs intensive study.

The NOD mouse is a commonly used spontaneous model for type 1 diabetes (2). In this model, the first recognized sign of autoimmunity is infiltration of APC in and around pancreatic islets (7,8). These APC include DC as well as Mϕ. $CD11c^+$ DC accumulate in and around female NOD islets from 3-7 weeks of age onwards. Simultaneously, $ER\text{-}MP23^+$ Mϕ, which are normally present in the connective tissue in the NOD pancreas, migrate to the periphery of the islets (8). This early accumulation of cells (of which the majority is presumably monocyte-derived) plays an essential role in the development of the insulitis, since prevention of the early influx of monocytes stops the process (9). Only after this early (peri-)islet accumulation of DC and Mϕ, large numbers of T and B lymphocytes are recruited to the site, i.e., in NOD females from the age of 7-10 weeks onwards. In the BioBreeding (BB) rat, another spontaneous animal model for type 1 diabetes, insulitis also starts with an accumulation of APC (10). The trigger for the early accumulation of DC and Mϕ in the prediabetic pancreas is still unknown.

Autoimmune diseases might be caused by aberrations in the function of the immune system as well as in the target gland (7,11). In the prediabetic NOD mouse, several changes in the function of the islets of Langerhans have been described. From 4 weeks of age, prediabetic NOD mice females show signs of islet hyperfunction as evidenced by higher basal blood insulin levels and a lower glycemia as compared to mice of several control strains (12,13). Glucose tolerance tests also showed a more rapid blood insulin peak and a faster drop in blood glucose levels in prediabetic NOD mice compared to controls. Evidence for an early islet abnormality also has a morphological basis, since we previously showed a higher frequency of hyperplastic islets ("mega-islets") in prediabetic NOD mice compared to non-diabetic control strains (14).

The question thus arises in what manner endocrine islet abnormalities are related to the development of autoimmunity. We asked whether an association exists between islet hyperplasia, as a morphological sign of islet abnormality, and the development of the inflammatory infiltrate. To address this question, we compared the development of islet hyperplasia (mega-islet development) in NOD and NOD*scid* mice. The latter lack mature T and B cells

due to the *scid* mutation, and thus neither develop lymphocyte insulitis nor diabetes (15). If mega-islet development would be caused by infiltrating lymphocytes, this hyperplasia would not be observed in NOD*scid* mice. In order to define the relationship between infiltrating cells and islet hyperplasia more specifically, we investigated a putative correlation between infiltrates and islet size in both NOD and NOD*scid* mice. Finally, we wondered whether the previously observed β cell hyperactivity in itself plays a role in the formation of mega-islets. For this purpose, we used prophylactic insulin treatment that is known to decrease insulitis and diabetes incidence in rodents (16). In vivo administration of insulin is known to down regulate endogenous insulin secretion (17-20). Prophylactic insulin treatment can thus be used as a tool to prevent the development of β cell hyperactivity and to study mega-islet development in the absence of such hyperactivity. However, prophylactic insulin also affects tolerance induction towards islet antigens (21). Therefore, we studied the effect of prophylactic insulin treatment on islet hyperplasia in NOD*scid* mice in addition to NOD mice. In this way, we exclude indirect effects of prophylactic insulin treatment on islet hyperplasia via lymphocytes. The results indicated that both islet hyperactivity in itself and infiltrating lymphocytes appear to be involved in the observed mega-islet formation.

Results

NOD and NOD*scid* mice both develop increased percentages of mega-islets, but this phenomenon is less prominent in the NOD*scid* mouse

To determine the kinetics of mega-islet development and the contribution of infiltrating cells, we first compared the development of islet sizes in NOD, NOD*scid* and C57BL/10 females at different ages (Fig. 1). Mega-islets were defined as islets with an area of more than 10000 pixels at a magnification of 100x.

At 5 weeks of age, the percentage of mega-islets was similar in the three strains (around 10% of the islets). Between 5 and 10 weeks of age, NOD and NOD*scid* females developed significantly increased numbers of mega-islets (i.e. between 15-20% of the islets), whereas, in C57BL/10 mice, the percentage of mega-islets remained constant (i.e., around 10% of the islets) ($p<0.05$ for both NOD and NOD*scid* vs. C57BL/10). At 15 weeks of age, NOD mice exhibited a further increase in the number of mega- islets, which reached over 20% of total islets. In contrast, in NOD*scid* mice, the percentage of mega-islets remained similar between 10 and 20 weeks of age (around 15-20% of the islets) ($p<0.05$ for NOD vs. NOD*scid*). In NOD mice over 15 weeks of age, islet size could not be measured because of the presence of destructive insulitis, a phenomenon that does not take place in NOD*scid* mice.

In conclusion, mega-islet formation takes place after 5 weeks of age in both NOD and NOD*scid* mice and appears to correlates in time with the progression of infiltration. However, the phenomenon is less prominent in NOD*scid* mice, suggesting a contribution of infiltrating lymphocytes to mega-islet formation.

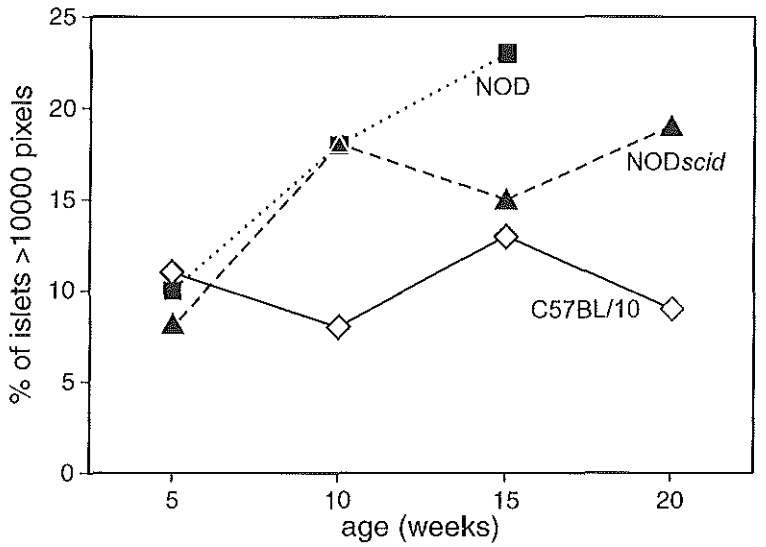

Figure 1: Development of mega-islets, as a function of age, in NOD, NOD*scid* and C57BL/10 mice (n=5 mice/age group). Results are percentages of total number of islets from 5 mice in each age-strain group.

Initial infiltration of DC, Mϕ and lymphocytes is predominantly associated with mega-islets, but is less pronounced in NOD*scid* mice

Since the start of mega-islet formation correlated in time with the initiation of a substantial leukocyte infiltration, we wanted to specify the relationship between mega-islet formation and leukocyte infiltration in more detail. Therefore, the infiltration of the islets in relation to their size was compared in NOD and NOD*scid* females at 6 weeks of age (the time at which there is a predominant infiltration of DC and Mϕ and, in NOD mice, only limited numbers of lymphocytes). The intensity of infiltration was measured in two ways.

First, the percentage of islets infiltrated with ER-MP23$^+$ Mϕ, CD11c$^+$ DC and lymphocytes was determined. CD11c$^+$ DC and lymphocytes both do not normally occur in the pancreas of control animals at this age. Hence, every positive cell in the pancreas was considered to be an infiltrating cell and thus an islet was defined infiltrated if at least one positive cell was situated around and/or within the islet. For ER-MP23$^+$ Mϕ, this definition is different, because ER-MP23$^+$ Mϕ are resident cells in the exocrine pancreas, but they are never localized in the islets of non-autoimmune prone control animals (8). Therefore, for ER-MP23$^+$ Mϕ, an islet was defined infiltrated only if at least one positive cell was situated within the islet, and not if positive cells were only present around the islet.

Second, the extent of infiltration per islet was determined by measuring the size of the infiltrate. For lymphocytes, this was done by measuring the size of the lymphocyte infiltrate, which is localized next to the islet at this age (lymphocytes rarely infiltrate the islet itself at

this age) (Fig. 2). For CD11c$^+$ DC and ER-MP23$^+$ Mϕ, this was done by measuring the percentage of islet surface positive for the CD11c or the ER-MP23 marker. In this way, APC infiltration is expressed as a density and thus corrected for the islet size that was measured.

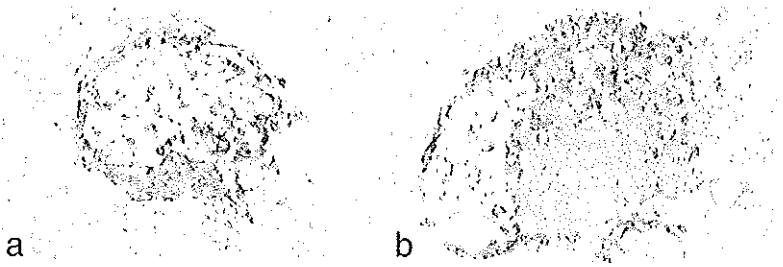

Figure 2: Development of peri-insulitis in an NOD female at 10 weeks of age. Staining for CD11c$^+$ DC, original magnification x250. Note that CD11c$^+$ DC already infiltrate the islet at stages in which lymphocyte accumulation around the islets is relatively limited (a). Lymphocytes, in contrast, form an infiltrate adjacent to the islet, even if already high numbers of lymphocytes are present (b).

CD11c$^+$ DC were preferentially associated with the mega-islets in 6-week-old NOD mice. The percentage of CD11c$^+$ DC-infiltrated islets was significantly higher in mega-islets compared to normal islets ($p<0.001$) (Fig. 3). It can, however, be argued that mega-islets have a higher chance of being infiltrated by an APC solely because of the bigger size of the area that is measured. Therefore, we also looked at the density of the islet infiltration with APC in normal islets and mega-islets by expressing the CD11c$^+$ surface area related to the total islet surface area. We found that the density of infiltration with CD11c$^+$ DC was significantly higher in mega-islets compared to normal islets ($p<0.0001$) (Table 1). To exclude the effects of lymphocytes, we determined DC-infiltration in NOD*scid* mice. In these mice, as observed in NOD mice, the percentage of CD11c$^+$ DC-infiltrated islets was significantly higher in mega-islets compared to normal islets ($p=0.01$) (Fig. 3). However, differences between NOD*scid* and NOD mice were observed: both the percentage of CD11c$^+$ DC-infiltrated islets (Fig. 3) and the density of CD11c$^+$ DC-islet infiltration (Table 1) were significantly reduced in NOD*scid* compared to NOD mice ($p=0.01$ and $p=0.048$, respectively).

Table 1: The density of infiltration with CD11c$^+$ DC and ER-MP23$^+$ Mϕ is higher in mega-islets as compared to normal islets in NOD and NOD*scid* mice at 6 weeks of age. Statistical analysis of the density of infiltration was done by the Mann-Whitney U Wilcoxon Rank Sum W test. This test orders the measurements and gives each measurement a rank number. The differences between the groups are than tested based on their mean rank number. Mean ranks of this percentage for each group as determined by this test as well as the range of measurements are depicted in this table.]$p<0.05$.

Strain		CD11c+ DC		ER-MP23+ Mϕ	
		Density range	Mean rank	Density range	Mean rank
NOD*scid*	Normal islets	0 – 0.25	16	0 – 2.2	19]
	Mega-islets	0 – 0.40	18	0 – 1.7	27]
NOD	Normal islets	0 – 2.4	34]	0 – 2.9	41]
	Mega-islets	0 – 2.1	55]	0 – 2.3	61]

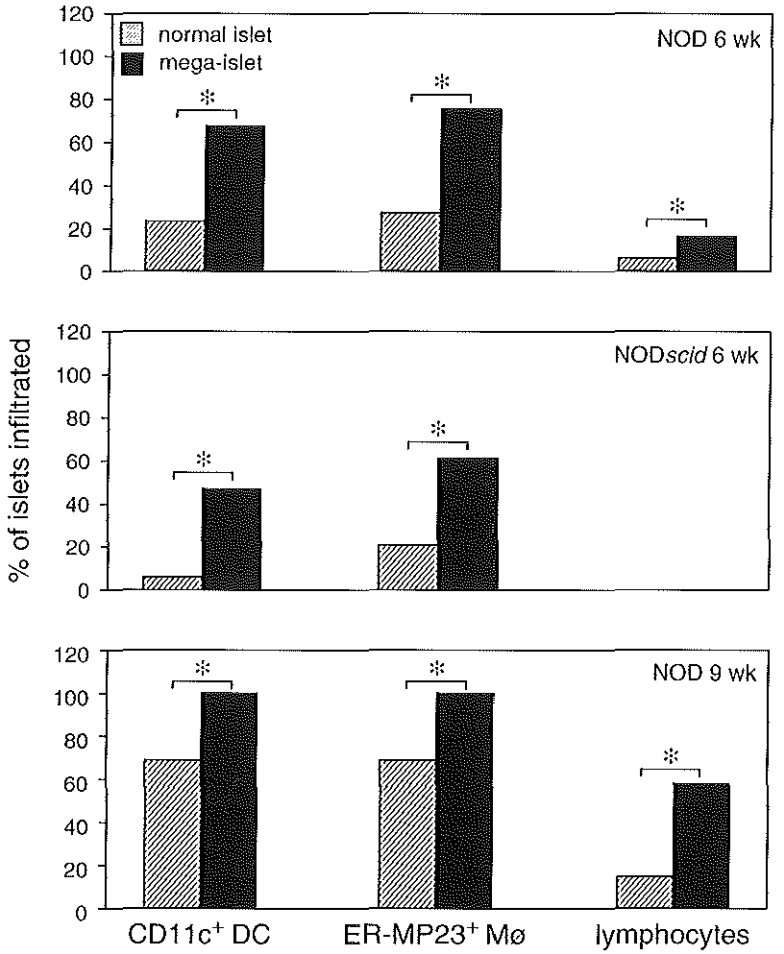

Figure 3: Compared to normal islets, mega-islets are preferentially infiltrated with early infiltrating APC and lymphocytes. An islet is defined as infiltrated with ER-MP23$^+$ Mϕ if there is at least one cell detected within the islet, and defined as infiltrated with CD11c$^+$ DC or lymphocytes if there is at least one cell present within or around the islet (see Result section). Results are percentages of total number of islets from 5 mice in each age-strain group.

ER-MP23$^+$ Mϕ also preferentially infiltrated mega-islets in NOD mice. The percentage of ER-MP23$^+$ Mϕ-infiltrated islets was significantly higher in mega-islets than in normal islets (p=0.00001) (Fig. 3). Furthermore, the density of ER-MP23$^+$ Mϕ infiltration in these islets was higher in mega-islets than in normal islets (p=0.0001) (Table 1). In NOD*scid* mice, the percentage of ER-MP23$^+$ Mϕ-infiltrated islets (Fig. 3) and the density of infiltration with ER-MP23$^+$ Mϕ (Table 1) were also significantly higher in mega-islets than in normal islets (p=0.01 and p=0.02, respectively). However, as observed for CD11c$^+$ DC, the percentage of ER-MP23$^+$ Mϕ infiltrated-islets (Fig. 3) was significantly lower in NOD*scid* compared to NOD mice (p=0.02).

Although lymphocyte infiltration was minor in 6-week-old NOD mice, lymphocytes were also preferentially situated around mega-islets. The percentage of islets surrounded by lymphocytes was significantly higher in mega-islets than in normal islets ($p=0.02$) (Fig. 3) and the area of the lymphocyte infiltrate was larger near the mega-islets as compared to the normal islets ($p=0.001$) (Table 2). Later, at 9 weeks of age, the percentages of infiltrated islets (Fig. 3) and the area of the lymphocyte infiltrate (Table 2) were significantly higher in mega-islets compared to normal islets ($p<0.001$ in both cases). As expected, lymphocytic infiltration was completely absent in NOD*scid* mice.

Table 2: The size of the lymphocyte infiltrate near mega-islets is higher than near normal islets in NOD mice of 6 and 9 weeks of age. Statistical analysis of the density of infiltration was done by the Mann-Whitney U Wilcoxon Rank Sum W test. This test orders the measurements and gives each measurement a rank number. The differences between the groups are than tested based on their mean rank number. Mean ranks of this percentage for each group as determined by this test as well as the range of measurements are depicted in this table.]$p<0.05$.

Strain	Age		Lymphocytes	
			Size range	Mean rank
NOD	6 weeks	Normal islets	0 – 8907	87]
		Mega-islets	0 – 28514	97]
NOD	9 weeks	Normal islets	0 – 59865	45]
		Mega-islets	0 – 53249	68]

In conclusion, the first infiltrating cells are preferentially found near the mega-islets. This is the case for both early infiltrating APC and lymphocytes. The association between mega-islets and early infiltrating APC can be found in NOD and NOD*scid* mice, although APC infiltration in NOD*scid* mice is relatively limited as compared to NOD mice.

Prophylactic insulin treatment prevents mega-islet formation in both NOD and NOD*scid* mice

Since β cell hyperactivity was increased before the appearance of mega-islets (7,13,14), we wondered whether it could be a contributing factor in their formation, in addition to immune cell infiltration. We thus assessed the effect of prophylactic insulin treatment, known to down-regulate β cell activity (17-19), in NOD and NOD*scid* mice. In the latter, lacking functional lymphocytes, we therefore avoided the tolerizing effects of insulin on lymphocytes (21). Figure 4 shows that prophylactic insulin treatment, administered from 3 to 6 weeks of age, decreased significantly the percentage of mega-islets in NOD mice ($p=0.003$), confirming previous data (14). Moreover, it also decreased this percentage in NOD*scid* mice to a similar extent ($p<0.001$). It should be noted, however, that the percentages of mega-islets in both groups of placebo-treated NOD and NOD*scid* mice were higher

than in untreated controls (Fig. 1). This increase in mega-islet frequency is most likely explained by the stress induced by repeated placebo injections, since an increased islet mass has been observed after repeated placebo injection (22).

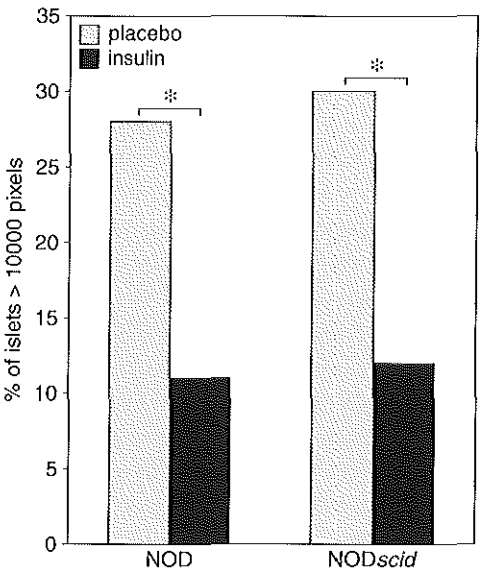

Figure 4: Prophylactic insulin treatment prevents mega-islet development in both NOD and NOD*scid* mice. NOD females and NOD*scid* females were subcutaneously treated from 3 weeks to 6 weeks of age, with insulin or diluent. Results are percentages of total number of islets from 8 mice in each group.

In conclusion, down regulating β cell activity by means of prophylactic insulin treatment reduces mega-islet formation in both NOD and NOD*scid* mice. This suggests that the islet activity in itself plays a role in initiating mega-islet formation.

Discussion

In this study, we show that the largest islets or mega-islets, which are observed more frequently in NOD and NOD*scid* mice than in controls, are preferentially associated with early infiltrating leukocytes. This concerns DC and Mϕ in both NOD and NOD*scid* mice, and lymphocytes in NOD mice. In NOD*scid* mice, mega-islet formation is less prominent than in NOD mice, suggesting that lymphocytes contribute to mega-islet formation. Moreover, β cell metabolic activity itself appears to play a role, since its down-regulation by an early and short prophylactic insulin treatment decreased the percentages of mega-islets in both NOD and NOD*scid* strains.

In this study, we show that NOD mice had a higher fraction of mega-islets than control C57BL/10 mice. In a previous study, we observed a comparable difference using Balb/c mice as a control strain (14). Moreover, our results on β cell hyperplasia in NOD and NOD*scid* mice are in agreement with studies showing a large β cell mass in prediabetic NOD mice or in insulitis-free NOD stocks (NOD*scid*, NOD-NON H-2_{nb1} and NOD $B2^{m}{}_{null}$ (23). Mega-islet formation in NOD mice is associated in time with the appearance of the first infiltrating cells. Both the start of the β cell hyperactivity (as assessed by increased blood insulin levels) and the initiation of infiltration with DC and Mϕ take place around 4 weeks of age (12,13). In BB rats, there are also indications for a prediabetic β cell hyperactivity in relation to immune infiltration. Earlier studies showed an enhanced β cell sensitivity to glucose and an increased insulin secretion by inflamed islets from non-diabetic BB-DP rats, compared to non-inflamed islets from the same or from a control strain (24). Therefore, a period of transient islet hyperactivity and hyperplasia associated with the initiation of infiltration appears to precede β cell exhaustion and β cell death in both the NOD mouse and the BB rat.

In addition to an association in time, there is also a relationship regarding their mutual location: mega-islets are preferentially associated with early infiltrating APC and lymphocytes. The association of APC infiltration and mega-islets could already be found at 6 weeks of age in NOD mice. At this age, the number of mega-islets in NOD mice is similar to that found in control mice. Since control mice do not exhibit leukocyte infiltration, it could be suggested that the NOD mega-islets function differently than the control ones. Interestingly, at this same age increased circulating insulin levels can be found in NOD mice (12,13).

Two major mechanisms could underlie the association between mega-islets and inflammatory infiltrates. First, the abnormal islet function could be the cause of APC infiltration, via, for example, insulin chemotaxis (25,26). Second, mega-islet formation could be the consequence of the inflammatory infiltrates, via the influence of released factors. In various other endocrine organs, leukocytes have been shown to influence endocrine cell growth and/or function (27-29). As discussed below, both mechanisms may be at play.

First, β cell hyperactivity could contribute to the mega-islet formation by attracting leukocytes. Since insulin is known to down regulate its own secretion (17-19) and to decrease pancreatic insulin content in NOD mice (20), we used it to prevent the transient β cell hyperactivity which we previously described from 4 to 8 weeks of age in mice with the NOD genetic background (12,13). Here, we confirmed that prophylactic insulin treatment is able to reduce mega-islet formation in NOD mice (14). However, since the treatment could act on lymphocytes, via the induction of tolerance to insulin (21), we also prophylactically treated NOD*scid* mice, which lack functional lymphocytes. Insulin treatment was able to prevent mega-islet formation in NOD*scid* mice as efficiently as in NOD mice. These results therefore suggest a role for β cell hyperactivity in mega-islet formation.

Second, we assessed whether leukocytes could influence mega-islet formation. The results obtained in NOD*scid* mice shows that this mechanism is also playing a role. Although both NOD and NOD*scid* females showed an early DC and Mϕ infiltration and an early hyper-

insulinism (12,13), later followed by the presence of mega-islets, nevertheless, clear differences were observed between the two strains. The first difference is the reduced infiltration of $CD11c^+$ DC and $ER\text{-}MP23^+$ Mϕ in NOD*scid* females. Lower levels of $CD11c^+$ and $F4/80^+$ cells were also suggested in another study (30), and the differences are thus quantified here. One explanation for this reduced APC infiltration might be related to the decreased function of APC in NOD*scid* mice. Indeed, APC isolated from NOD*scid* islets are much less effective in stimulating diabetogenic T cell clones than those isolated from NOD islets. Transfer of diabetogenic T cell clones in vivo improves the antigen-presenting function of NOD*scid* islet APC (31). Thus, lymphocytes and/or their products regulate the function and phenotype of APC. The second difference between the two strains is that NOD*scid* females did not only show a reduced APC infiltration, but also showed a less pronounced hyperinsulinism (13) and fewer mega-islets. Indeed, mega-islet formation is higher in NOD than in NOD*scid* mice only after 10 weeks, i.e., a few weeks after the beginning of the lymphocyte infiltration that then regularly progresses. These data, therefore, suggest an in vivo stimulatory effect of the lymphocyte compartment on islet cell function and growth in NOD mice.

In conclusion, a close association is shown for the first time between early target organ abnormalities and the initiation of leukocyte infiltration in a spontaneous model of type 1 diabetes. The precise sequence of cellular events is still unknown, but the differences between NOD strains (NOD and NOD*scid*) and control strains show that some inherent degree of β cell hyperactivity exists. It has been suggested previously that a period of islet hyperactivity may be crucial in the development of autoimmunity (7). Hyperactive β cells are more prone to autoimmune reactions because of high levels of autoantigens, adhesion and MHC molecules and because of a higher sensitivity to cytokine-induced damage (7,32-34). The importance of the target gland abnormality for diabetes development is also indicated by the fact that both hyperinsulinemia and mega-islet formation are more pronounced in diabetes-prone NOD females compared to less diabetes-prone NOD males. In line with this, orchidectomy of NOD males leads to increased percentages of mega-islets and a higher diabetes incidence (manuscript submitted). This initial target organ abnormality could attract APC (and lymphocytes in NOD mice), which in turn cause a further stimulation of islet activity. Such a view is supported by the association of infiltrates and mega-islets and by the differences in mega-islet formation between NOD mice (with high APC and lymphocyte infiltration) and NOD*scid* mice (with limited APC and no lymphocyte infiltration). The resulting β cell exhaustion and/or destruction would trigger the b cell activity of other still unaffected islets, in order to maintain normal blood glucose levels. Thus, a vicious circle may have started resulting in clinical onset of diabetes.

Materials and methods

Animals

NOD, NOD*scid* and C57BL/10 female mice were bred under specific pathogen-free conditions at the facilities of the Hôpital Necker, Paris, France. The animal facilities and care followed the norms stipulated by the European Community. The incidence of diabetes in the NOD colony is, by 200 days of age, 80% for females and 40% for males. Mice were killed at 5, 10, 15 or 20 weeks of age for the assessment of islet size without any treatment, and at 6 weeks of age for the evaluation of the DC, Mϕ and lymphocyte infiltration in relation to islet size after treatment (placebo or insulin).

Insulin treatment

Sixteen NOD females and sixteen NOD*scid* females were treated from 3 weeks (weaning) to 6 weeks of age. In this group, eight NOD females and eight NOD*scid* females were treated with 0.25 U Protamine-Zinc-insulin (Organon, Oss, The Netherlands) per day, eight mice from each strain served as controls and were treated with the same volume of vehicle (placebo-treated group). All mice were weighed and assessed for glycemia at 3 and 6 weeks of age. Prediabetic NOD mice (with basal nonfasting glycemia < 11 mmol/l, as assessed by Glukotest, Boehringer-Mannheim, Mannheim, Germany) were used only for immunohistochemical analyses.

Antibodies

ER-MP23, identifying mouse macrophage galactose specific $(MMGL)^+$ MHC-class II^+ Mϕ (8,35), was used as a rat-anti-mouse hybridoma culture supernatant. N418, identifying $CD11c^+$ DC (35), was used as a hamster-anti-mouse hybridoma culture supernatant. β cells were identified by guinea-pig anti-porcine-insulin polyclonal antibody (Dako Glostrup, Denmark), which was used diluted 1:250 in phosphate buffered saline (PBS) with 0.1% Tween (Merck; Darmstadt, Germany) (PBS/Tween). ER-MP23 was detected with horseradish peroxidase (HRP)-conjugated rabbit-anti-rat immunoglobulins (Dako). N418 was detected with HRP-conjugated goat-anti-hamster immunoglobulins (Jackson ImmunoResearch Laboratories, Inc.; West Grove, Pennsylvania, USA). Anti-insulin was detected with HRP-conjugated rabbit-anti-guinea pig immunoglobulins (Dako).

Immunohistochemistry

Mice were killed by cervical dislocation after rapid retro-orbital bleeding. Their pancreases were removed, embedded in OCT (Sakura, Zoeterwoude, The Netherlands) compound and frozen in n-hexane on dry-ice chilled alcohol. Tissues were stored at -80°C until immunohistochemistry was performed. Before sectioning, microscopic slides were coated with a solution of 0.1% gelatine/0.01% chromium-alum. After a wash with phosphate-buffered saline with 0.1% Tween-20 (Merck, Schuchardt, Germany) (PBS/Tween), slides were incubated with first step monoclonal antibodies for 30 min at room temperature in a moist chamber. Subsequently, slides were washed with PBS/Tween and incubated with second step antibodies in the presence of 2% normal mouse serum for 30 min at room temperature. After an additional wash with PBS/Tween, slides were incubated with 0.05% (wt/vol) Ni-di-amino-benzidine (Ni-DAB) (Sigma, St. Louis, USA) with 0.02% H_2O_2 and washed in PBS/Tween after 3 min. Finally, slides were counterstained for 3 min in nuclear fast red, washed in water, dehydrated in a graded ethanol series, and mounted. For each series of pancreas sections, one slide was stained with second antibody only as a control for endogenous peroxidase activity and non-specific binding of the second step. A section of spleen was included as a positive control.

Quantification of immunohistochemistry

The surface area of the islets and the infiltrate as well as the percentage of islet surface positive for $CD11c^+$ DC or $ER\text{-}MP23^+$ Mϕ were assessed via a VIDAS-RT image analysis system (Kontron Elektronik GmbH/ Carl Zeiss, Weesp, the Netherlands). Measurements were performed using a magnification of 100x. Results of measurements were expressed in pixels. At a magnification of 100x, the size of one pixel is 1.13×10^{-6} mm^2. For the measurement of islet sizes, sections were stained with anti-insulin and a nuclear fast red counterstaining. For all measurements, islets were encircled by the researcher in such a way that the whole islet was measured and not only the area positive for insulin. For the measurement of APC infiltration, sections were stained with N418 or ER-MP23 and a nuclear fast red counterstaining.

Statistical analysis

Statistical comparison of the percentage of mega-islets between groups was carried out using the X^2-Test. The Mann-Whitney U Wilcoxon Rank Sum W Test was used to determine statistical significance for differences in the absolute amount of infiltration and the X^2-Test for differences in the percentage of large islets and the percentage of infiltrated islets. The level of significance was set as $p < 0.05$. For measurements of islet size, a mean of 190 islets was analyzed for every age-strain group of 5 mice. For measurements of infiltration, a mean of 60 islets was analyzed for every age-strain group of 5-8 mice.

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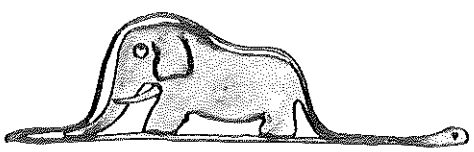
Chapter 3.2

Sex steroids influence pancreatic islet hypertrophy and subsequent autoimmune infiltration in NOD and NOD*scid* mice



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Summary

Female nonobese diabetic (NOD) mice more frequently develop autoimmune diabetes than NOD males. Orchidectomy of the latter aggravates insulitis and diabetes. Since clear differences in immune function have not been observed between prediabetic females and males, before or after castration, we hypothesized that sex-related differences in diabetes incidence are related to target organ-specific actions of sex steroids. Previously, we showed that prediabetic NOD females develop hyperinsulinemia and subsequently mega-islets. Infiltration of the first inflammatory leukocytes is predominantly associated with these mega-islets. Here, we determined the relationship between sex hormones, mega-islet formation and infiltrating cells in NOD and NOD*scid* mice.

Mega-islet formation was reduced in NOD males compared to NOD females and orchidectomy increased it, indicating a relationship between androgen levels and mega-islet formation. Moreover, enhanced mega-islet formation in castrated NOD males was associated with increased numbers of infiltrating leukocytes. Castrated NOD*scid* males also exhibited increased mega-islet formation and dendritic cell infiltration, indicating that lymphocytes are not required for castration-induced effects.

In conclusion, we show that androgens influence pancreatic islets and subsequent autoimmune infiltration in NOD and NOD*scid* mice. This suggests that the gender difference in diabetes incidence in NOD mice is related to target organ-specific androgen effects.

Introduction

The sexual dimorphism of autoimmune diseases - females have a higher incidence than males - is well known in both humans and animal models (1-4). This difference is generally assumed to be caused by the effects of sex steroids on the immune response, particularly the immunosuppressive effects of androgens (2,4-6).

Also in the nonobese diabetic (NOD) mouse model of type 1 (insulin-dependent) diabetes mellitus (type 1 diabetes), females become diabetic earlier and in higher frequency than males. Castration of males leads to an incidence comparable to that observed in females, whereas treatment with androgens prevents the development of diabetes in female NOD mice (7,8). It is generally assumed that the autoimmune process in NOD females and castrated males progresses due to the absence of immunosuppressive androgens. However, data comparing various immunological parameters between NOD females and males, and between castrated and control animals of both sexes, have not given conclusive evidence to support such a view. Splenic T cell proliferation, IL-2 production and antibody production against sheep red blood cells do not reveal any spontaneous sex difference or any effect of castration, while leukocyte phenotype analysis showed only minor differences (7). In addition, autoimmune inflammatory infiltration is not always predominant in females: while a NOD female preponderance exists for the development of insulitis and sialoadenitis (7,9), dacryoadenitis is preferentially found in NOD males (10). Taken together, these observations in NOD mice question the general view that sex steroid effects on the immune system explain the observed gender difference in diabetes incidence. Alternatively, sex hormones may directly affect the target organ and thus influence the susceptibility for autoimmune attack.

In the NOD pancreas, the first sign of autoimmunity is an early accumulation of macrophages (Mϕ) and dendritic cells (DC) around the islets of Langerhans (peri-insulitis) (11,12). These cells are assumed to function as antigen-presenting cells (APC). Subsequently, numerous T and B lymphocytes are recruited to the site and the progressive insulitis leads to the destruction of the insulin-producing β cells located in the core of the islet of Langerhans. In the NOD*scid* mouse, an early peri-islet APC accumulation also takes place, but to a lesser extent (13). Due to the lack of functional lymphocytes, however, neither lymphocyte insulitis nor diabetes develops in these mice (14). With age, both NOD and NOD*scid* females exhibit increased numbers of large-sized islets ('mega-islets') (12,15). The importance of these mega-islets for diabetes development is indicated by the observation that the early infiltrating APC are predominantly situated next to these mega-islets in both strains (12,15). This is particularly the case for $CD11c^+$ dendritic cells (DC).

The causes of mega-islet development are not known. Islet hypertrophy occurs consecutively to endocrine abnormalities, suggesting a role for these abnormalities in mega-islet development. More specifically, a transient β cell hyperactivity is observed in NOD mice, particularly females, that is closely associated in time to the emergence of APC infiltration

(16,17). Interestingly, an aberrant sexual dimorphism in insulin response to a glucose load exists in NOD mice: females produce, as early as 6 weeks of age, higher insulin levels than males (16). This glucose response pattern is opposite to the one found in normal rodents (16). Moreover, insulitis progresses more rapidly in NOD females than in males, possibly as a consequence of this β cell hyperactivity (16-18). Thus, functional endocrine abnormalities in NOD mice precede the formation of mega-islets and subsequent autoimmune infiltration. Moreover, these abnormalities seem to be especially marked in diabetes-prone NOD females.

We hypothesized that the sensitivity for autoimmune attack as observed in NOD females was related to sex steroid effects on the target organ instead of on the immune system. Therefore, we have studied the effect of castration on the islet of Langerhans by measuring early mega-islet development, APC infiltration and the relationship between these two parameters. By using both NOD and NODscid mice, we determined the contribution of lymphocytes to islet abnormalities developing upon castration.

Results

A gender bias in mega-islet formation exists in NOD and NOD*scid* mice

Previous studies showed early hypertrophy of pancreatic islets, leading to 'mega-islet' formation in NOD and NOD*scid* female mice (12,15). To assess a possible sexual dimorphism in mega-islet formation, we measured islet size in NOD and NOD*scid* mice of both sexes at 5, 10 and 15 weeks of age. Mega-islets were defined as islets with an area of more than 10000 pixels at a magnification of 100x.

Five-week-old females and males of both strains had comparable percentages of mega-islets (Fig. 1). At 10 weeks of age, however, significant differences in the percentages of mega-islets were observed between females and males regardless of the strain. NOD and NOD*scid* females had higher percentages of mega-islets than NOD and NOD*scid* males ($p<0.05$ for NOD females vs. NOD males; $p<0.05$ for NOD*scid* females vs. NOD*scid* males). However, after the appearance of this sexual dimorphism in mega-islet development in both NOD and NOD*scid* mice, a strain difference developed, as observed at 15 weeks of age. Significantly higher percentages of mega-islets were observed in NOD females and males compared to sex-matched NOD*scid* mice ($p<0.05$ for NOD females vs. NOD*scid* females; $p=0.05$ for NOD males vs. NOD*scid* males). This suggests that lymphocytes play a role in later phases of mega-islet development in NOD mice.

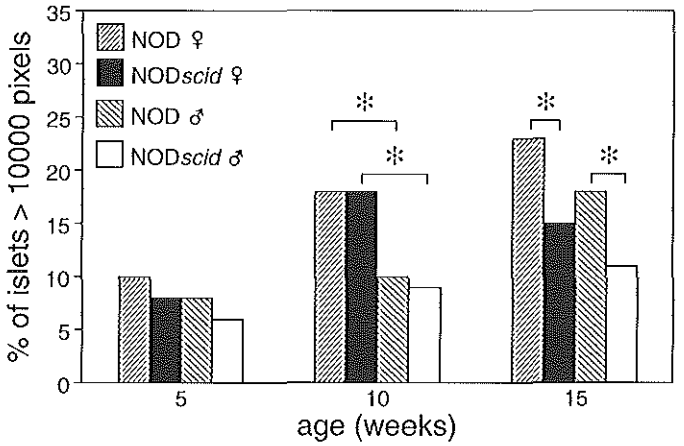

Figure 1: Development of mega-islets as a function of age in NOD and NOD*scid* females and males. Results are percentages of total number of islets from 5 mice. Note a significant difference between males and females at 10 weeks of age, and a significant difference between NOD and NOD*scid* mice, regardless of the sex, at 15 weeks of age.

Castration enhances mega-islet formation in NOD and NOD*scid* males and increases diabetes incidence in NOD males

As shown in Figure 2, orchidectomy of NOD and NOD*scid* mice at 4 weeks of age significantly increased the fraction of mega-islets at 10 weeks of age ($p<0.001$ for NOD males; $p<0.05$ for NOD*scid* males). This increase is especially marked in NOD males, leading to a significant difference between orchidectomized NOD and NOD*scid* males ($p<0.001$).

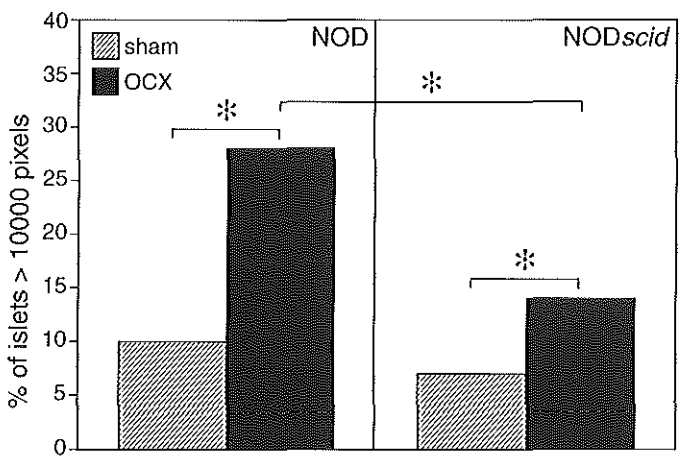

Figure 2: Presence of mega-islets in orchidectomized and sham-operated 10-week-old NOD and NOD*scid* males. Results are percentages of total number of islets from 6-8 mice/age group. Orchidectomy results in a rise in the percentage of mega-islets. Moreover, this effect is significantly higher in NOD compared to NOD*scid* males.

We also confirmed previous findings that orchidectomy at 4 weeks of age significantly increased diabetes prevalence in NOD males (data not shown).

Castration enhances DC infiltration around and/or into the islets of NOD and NOD*scid* males

Since $CD11c^+$ DC accumulate around as well as within the islet (Fig. 3), we measured infiltration in the peri-islet area and in the islet itself at 10 weeks of age (for details: see Materials and Methods section).

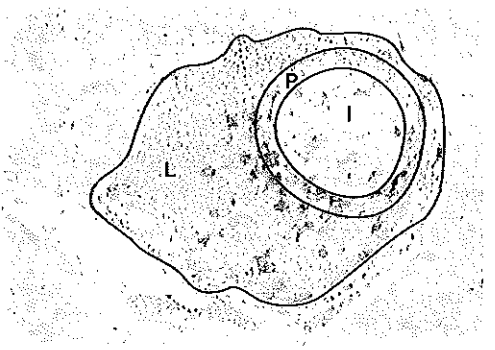

Figure 3: The location of $CD11c^+$ DC (black) and lymphocytes in islet infiltrates (NOD female, 10 weeks old). I: islet; P: peri-islet area; L: lymphocyte infiltrate. Original magnification x64.

Figure 4 shows that orchidectomy in NOD mice enhanced the accumulation of $CD11c^+$ DC in both the islet itself (4.4 vs. 1.1 % of the islet surface area was occupied by DC in the orchidectomized vs. sham-operated group, $p<0.0001$) and in the peri-islet area (19.2 vs. 6.7 % for orchidectomy vs. sham-operated, $p<0.0001$). In NODscid mice, the accumulation of $CD11c^+$ DC was also significantly enhanced in the peri-islet area after orchidectomy (2.7 vs. 1.4 % for orchidectomy vs. sham-operated, $p<0.001$), but in the islet itself, the difference did not reach statistical significance (0.38 vs. 0.25 % for orchidectomy vs. sham-operated).

NOD and NOD*scid* mice differed significantly with regard to peri-islet or intra-islet $CD11c^+$ DC infiltration (Fig. 4). In sham-operated NOD males, $CD11c^+$ DC infiltration was more extensive than in NOD*scid* males, both in the islet itself (1.1 vs. 0.25 % for NOD vs. NOD*scid*, $p=0.0001$) as well as in the peri-islet area (6.7 vs. 1.4 %, $p<0.001$). After orchidectomy, this difference increased both in the islet itself (4.4 vs. 0.38 %, $p<0.0001$) as well as in the peri-islet area (19.2 vs. 2.7 %, $p<0.0001$). This suggests that lymphocytes are likely to play a role in the extent of peri- and intra-islet DC infiltration in the NOD mouse.

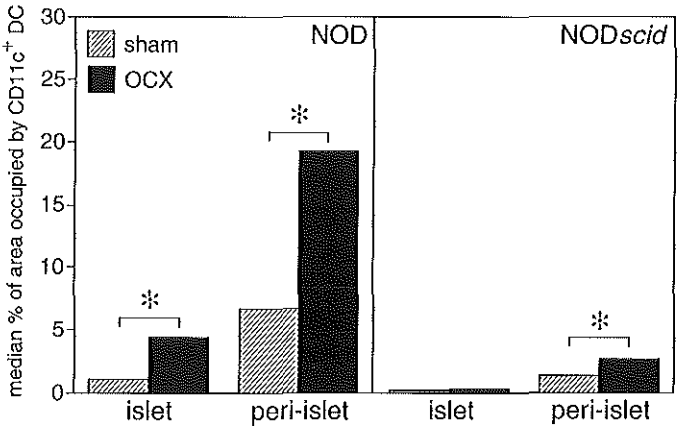

Figure 4: $CD11c^+$ DC infiltration in orchidectomized and sham-operated 10-week-old NOD and NOD*scid* males. Infiltrating DC are distinguished with regard to their location: with the islets or in the peri-islet inflammatory infiltrate. The amount of infiltrating cells is expressed relative to the islet or peri-islet surface area (see Materials and Methods). Note that orchidectomy of NOD and NOD*scid* males leads to an enhanced accumulation of $CD11c^+$ DC around the islets. In NOD mice, orchidectomy also leads to an enhanced infiltration of $CD11c^+$ DC into the islets. As expected for this age, peri-islet infiltration is significantly higher than the infiltration of the islet itself. Moreover, $CD11c^+$ DC infiltration in NOD males is significantly higher as compared to that in NOD*scid* males.

Castration tends to enhance islet-related lymphocyte infiltration in NOD males

Figure 5 shows that orchidectomy of 4-week-old NOD mice increased the lymphocyte infiltration, when measured at 10 weeks of age. Indeed, the median size of the lymphocyte infiltrate was 856 pixels for sham-operated NOD males and 2398 pixels for orchidectomized NOD. However, this difference tended only toward significance (p=0.088). As expected, NOD*scid* mice did not exhibit any lymphocyte infiltration.

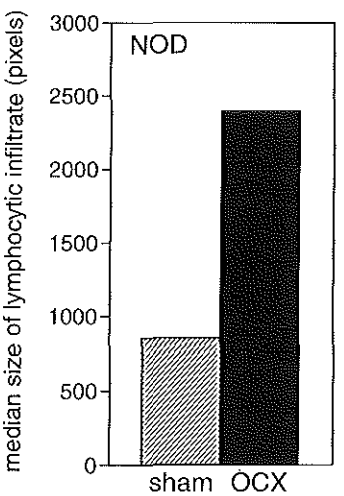

Figure 5: Development of lymphocyte infiltration in orchidectomized and sham-operated 10-week-old NOD males. Orchidectomy tended to increase the size of the lymphocyte infiltrate (p=0.088).

Discussion

In the NOD mouse model of type 1 diabetes, females are more prone to diabetes development than males, despite the absence of clear differences in peripheral immune function between both sexes (7). Similarly, castrated males show an enhanced diabetes incidence without obvious changes in peripheral immune function (7). Therefore, we hypothesized that a target organ-specific effect of sex steroids is operative in diabetes development. This study shows that orchidectomy induces early islet abnormalities in male NOD mice, indicated by the increase in mega-islet formation in castrated animals. Simultaneously, orchidectomy enhances early APC infiltration in NOD and NOD*scid* mice. The effect of orchidectomy on both mega-islet formation and APC infiltration is stronger in NOD than in NOD*scid* mice, indicating that lymphocytes enhance the effect of androgen deprivation.

The relationship between sex steroids, mega-islet formation and leukocyte infiltration can be explained in different ways. On the one hand, the effects of orchidectomy on the development of early islet abnormalities in male NOD mice may be achieved via a direct influence of sex steroids on islet cells and/or glucose homeostasis. On the other hand, sex steroids may affect the function of leukocytes that accumulate around and in the islets, and thereby cause islet abnormalities. The data presented here and those from the literature suggest that both mechanisms may be at work.

There are several indications that the islet itself can respond to sex hormones. Functional studies suggest that sex steroids might directly and/or indirectly influence islet function in normal animals. There is a sexual dimorphism in glucose homeostasis in normal rodents, with males showing higher nonfasting basal glycemia and insulinemia than females (16,19). This difference is related to the opposite effects of androgens and estrogens on insulin sensitivity of cells: androgens lower insulin sensitivity, while estrogens increase it (20-22). Accordingly, partial pancreatectomy-induced diabetes in normal rodents is more frequent in males than in females and its effect is reversed, in each sex, by castration (23). Finally, estrogen administration in rodents and orchidectomy in various species have been described to induce β cell hyperplasia and/or islet hypertrophy (24-26). Thus, our finding in NOD mice that orchidectomy led to an increased percentage of mega-islets in prediabetic NOD males (before any noteworthy lymphocyte infiltration) and in NOD*scid* males is fully in line with the earlier observations in other strains and species that sex steroids influence β cell function.

Do sex steroids act directly at the β cell level through specific receptors? Estrogen and progesterone receptors are indeed present in β cells (27-30). However, to the best of our knowledge, androgen receptors have not been shown in β cells. In addition, we ourselves were unable to detect the presence of these receptors in β cells (unpublished observation). These data make a direct effect of androgens at the β cell level unlikely.

If indeed β cells lack androgen receptors, is there an alternative explanation for the observed gender difference in diabetes incidence and the effect of orchidectomy in NOD mice? Sex steroids also have recognized effects on leukocyte function. It could be argued that sex steroids induce prediabetic islet abnormalities via an effect on leukocytes, either lymphocytes or APC, which accumulate around the islets.

We show here that orchidectomy affects mega-islet formation and APC infiltration in lymphocyte-deficient NOD*scid* mice. This indicates that at least part of the sex steroid effect, if achieved indirectly, can be mediated via non-lymphoid cells. APC are likely candidates as mediators of sex steroid effects. In general, a close physical and functional interaction between the endocrine system and APC has been observed for virtually all endocrine organs (31,32). In the thyroid, pituitary and the gonads, DC and macrophages play an important role in the regulation of hormone secretion (31,32). An additional study also suggests possible effects of APC on islets and a role of sex steroids in this interaction. In mice that express interferon-γ as a transgene in their β cells, interferon-γ-stimulated duct cell proliferation is stronger in females than in males. However, if the pancreatic influx of Mϕ that occurs in these mice is prevented, the proliferation in males is increased until the level found in females. Thus, interferon-g stimulation of duct cell proliferation is mediated part trough the Mϕ, which inhibits such growth in males (33). In conclusion, APC appear to be important communicators in the interactions between the endocrine and the immune system. How could sex steroids influence the interactions between APC and islet cells? Sex steroids are known to modulate cytokine production by leukocytes, in particular by mononuclear phagocytes (6). Furthermore, APC are able to convert pro-hormones to active androgens (34). Taken together, these notions support the view that sex steroids influence APC function and thus might, via these cells, also influence the islet cells.

The effects of orchidectomy on mega-islet formation are stronger in NOD males than in NOD*scid* males, however. This suggests that lymphocytes also contribute to mega-islet formation in NOD mice. A direct lymphocyte-mediated effect of changes in androgen levels, however, is unlikely, since mature lymphocytes lack androgen receptors (6). How then can the reduced effect of castration on mega-islet formation in NOD*scid* mice compared to NOD mice be explained? Androgens might act indirectly on lymphocytes by influencing products secreted by other cells present in the islet environment (35). Moreover, circulating estrogens, which persist in NOD males after castration, may have direct and similar immunostimulatory effects on lymphocytes from NOD females and castrated males, not counterbalanced by the presence of androgens (1,5,6). Also a role for infiltrating APC in this process can be envisaged. NOD*scid* have a reduced DC infiltration compared to NOD mice (this report for males, (13,15) for females). Therefore, the difference between NOD and NOD*scid* mice in mega-islet formation upon orchidectomy could be explained via effects on different numbers of infiltrating DC.

In conclusion, both leukocytes and islet cells themselves appear to play a role in the formation of mega islets after castration. In this experimental setting as well as in spontaneous disease, β cell hyperactivity and islet hypertrophy might be crucial in the development of dia-

betes. Hyperactive islets are known to express elevated levels of cell adhesion ligands, major histocompatibility complex (MHC) molecules and autoantigens, which may enhance the β cell-sensitivity to the cytotoxic effects of cytokines (18). We have previously demonstrated that prophylactic insulin treatment, which prevents insulitis and diabetes incidence, decreases mega-islet formation (12). Here we show that orchidectomy is associated with an increase of both mega-islet formation and incidence of diabetes in NOD mice. Moreover, orchidectomy enhances the early accumulation of $CD11c^+$ DC around and in the islets of NOD mice and NOD*scid* mice concomitantly with mega-islet formation. Together, these studies support the view that mega-islets are closely associated with and possibly functionally involved in the development of insulitis and subsequently diabetes in NOD mice. Since NOD*scid* mice lack functional lymphocytes, the stage of destructive insulitis is not reached in this strain.

Taken together, in this report we highlight the notion that sex steroids are important factors in the complex interrelationship between the islets of Langerhans and early infiltrating leukocytes in mice with the NOD genetic background. Sex steroids influence early prediabetic morphological islet abnormalities, as orchidectomy increases mega-islet formation and associated APC infiltration. This effect is enhanced by the presence of functional lymphocytes. In addition, an effect of androgens on islet cells either directly or indirectly via infiltrating APC, provides an explanation for the observed gender difference in diabetes incidence in NOD mice.

Materials and Methods

NOD colony, diabetes incidence and orchidectomy

NOD and NOD*scid* mice were bred under specific pathogen-free conditions at the facilities of the Hôpital Necker, Paris, France. The animal facilities and care followed the norms stipulated by the European Community. Mice were fed standard pellets and water ad libitum and were maintained at 22° C on a 12h light dark cycle. The incidence of diabetes in the NOD colony is 80% for females and 40% for males by 200 days of age.

NOD and NOD*scid* males were divided into 2 experimental groups: the first group underwent either orchidectomy under avertin anesthesia at 4 weeks of age, the second group was sham-operated to exclude the nonspecific effects of the operation itself. Mice were routinely checked for success of orchidectomy. One week after orchidectomy of NOD mice, circulating testosterone levels, assessed according to reference 17, had fallen from 12±2.1 ng/ml to 35.1±9.5 pg/ml (n=12). At the same time, circulating estradiol did not fall significantly: 63±13 pg/ml versus 46±10.5 pg/ml (n=9). For diabetes incidence assessment, 15 mice were included in each group. Animals were considered diabetic when glycemia was higher than 11 mmol/l using Glukotest (Boehringer-Mannheim, Mannheim, Germany).

To assess the effect of castration on immunohistochemical parameters (islet size and infiltration with $CD11c^+$ DC and lymphocytes), mice were used at 10 weeks of age (8/group). Unmanipulated NOD and NOD*scid* females and males were also used at 5, 10 and 15 weeks of age for the measurement of islet size (5 mice/group).

Antibodies

Islets were identified by guinea-pig anti-insulin polyclonal antibody (Dako, Glostrup, Denmark), which was used diluted 1:250 in phosphate buffered saline (PBS) with 0.1% Tween-20 (Merck-Schuchardt, Hohenbrunn bei München, Germany) (PBS/Tween). Anti-insulin binding was detected with HRP-conjugated rabbit-anti-guinea pig immunoglobulins (Dako). N418, identifying $CD11c^+$ DC (18), was used as a hamster-anti-mouse hybridoma culture supernatant. N418 was detected with HRP-conjugated goat-anti-hamster immunoglobulins (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania, USA). Lymphocyte infiltrates were identified morphologically by means of a nuclear fast red counterstaining (Fluka, Buchs, Switzerland).

Immunohistochemistry

Mice were killed by cervical dislocation after bleeding from the orbital sinus under ether anesthesia. Pancreases were removed, embedded in O.C.T. compound (Sakura, Zoeterwoude, The Netherlands) and frozen in dry ice-chilled isopentane for immunohistochemical studies. Tissues were stored at -80°C until immunohistochemistry was performed. Before sectioning, microscopic slides were coated with a solution of 0.1% gelatine/0.01% chromium-alum. Pancreas cryostat sections of 6 mm were prepared from central regions of the pancreas that included sufficient numbers of islets. Only one section of each pancreas was stained and measured to assure that an individual islet is not examined more than once. Sections were fixed for 2 min in 2% hexazotized pararosaniline (Sigma, St. Louis, USA) (36). After a wash with phosphate-buffered saline with 0.1% Tween-20 (PBS/Tween), slides were incubated with first step monoclonal antibodies for 30 min at room temperature. Subsequently, slides were washed with PBS/Tween and incubated with second step antibodies in the presence of 2% normal mouse serum for 30 min at room temperature. After an additional wash with PBS/Tween, slides were incubated with 0.05% (w/v) Ni-di-amino-benzidine (Ni-DAB) (Sigma, St. Louis, USA) with 0.02% H_2O_2 and washed in water after 3 min. Finally, slides were counterstained for 3 min in nuclear fast red, dehydrated in a graded ethanol series, and mounted. For each series of islet sections, one slide was stained with second antibody only as a control for endogenous peroxidase activity and nonspecific binding of the second step. A section of spleen was included as a positive control.

Quantification of immunohistochemistry

Total and marker-positive surface areas of infiltrated islets were assessed via a VIDAS-RT image analysis system (Kontron Elektronik GmbH/ Carl Zeiss, Weesp, The Netherlands). Measurements were done at a magnification of 100x. The results of the measurements were expressed in pixels. At a magnification of 100x, the size of one pixel is $1.13x\ 10^{-6}\ mm^2$.

For the measurement of islet sizes, islets were identified by means of an anti-insulin staining. For the measurement of infiltration, islets, peri-islet areas and lymphocyte infiltrates were recognized morphologically and encircled by the investigator. Figure 3 shows the definition of the islet area (I), the peri-islet area (P) and the lymphocyte infiltrate (P).

$CD11c^+$ DC are present mainly in peri-islet location, but also within the islet. Therefore, $CD11c^+$ DC infiltration in the peri-islet and islet areas was measured and expressed relative to peri-islet and islet area size, respectively. For lymphocyte infiltrates, infiltrates were expressed as absolute infiltrate sizes in pixels, since lymphocytes are not yet infiltrating the islets at the age under investigation but rather form a cuff at the side of the islet. The size of this cuff is directly related to the number of lymphocytes present. Therefore, it is not necessary to express lymphocyte infiltration relative to islet size. Thus, DC infiltration is expressed in a relative manner, whereas lymphocyte infiltration is expressed absolutely.

For all measurements, the results were analyzed for the total number of islets in a given experimental group, and not per individual mouse. The reason for this was that there was an extensive variability in the progression of the disease, hence in the number of islets available for analysis prior to destruction. A mean of 170 islets was analyzed for every age-strain group of 5 to 8 mice.

Statistical analysis

The X^2-Test was used to analyze differences in the prevalence of diabetes at 23 weeks of age between the orchidectomized and sham-operated mice. Also statistical comparison of the percentage of mega-islets between groups was carried out using the X^2-Test. Statistical comparison of the amount of infiltration with $CD11c^+$ DC and of the amount of lymphocyte infiltration between groups was carried out by the Mann-Whitney U-Wilcoxon rank Sum W Test. The level of significance was set as $p<0.05$.

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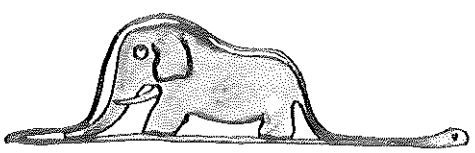
Chapter 3.3

Increased β cell activity in neonatal nonobese diabetic mice: *in situ* hybridization analysis of preproinsulin transcriptional levels



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Summary

Abnormalities of immune function are not sufficient to explain the organ/cell specificity of an autoimmune disease, for example β cells in diabetes. Among defects existing at the islet level in prediabetic nonobese diabetic (NOD) mice, we previously described a transient hyperinsulinemia followed by evidence of β cell hyperplasia. A modified *in situ* hybridization technique was used to determine if these effects were accompanied by changes in insulin transcriptional activity.

We found that NOD neonates express higher levels of preproinsulin primary transcript than age-matched C57BL/6 mice. To manipulate insulin transcriptional activity, NOD mothers were treated with insulin during the last two weeks of gestation. We observed a down-regulation of β cell hyperactivity in female neonates only. By contrast, the same insulin treatment applied to NOD*scid* mothers, devoid of functional lymphocytes, increased β cell activity.

In conclusion, mice with the NOD genetic background appear to have a relatively high level of β cell activity, reflected first in insulin transcriptional activity, that precedes any evidence of islet abnormalities or autoimmunity and may involve both genetic and environmental (maternal and fetal) factors.

Introduction

The NOD mouse is the most widely used animal model for type 1 (insulin-dependent) diabetes mellitus (type 1 diabetes) (1,2). Despite extensive research, however, the etiology of the disease is still largely unknown. In NOD mice, as in human (pre)diabetic patients, several abnormalities of immune function exist. These abnormalities, which include aberrant phenotypes and/or functions of thymocytes, antigen-presenting cells (APC), and lymphocytes, may hamper tolerance induction leading to autoimmunity (3-5). However, similar abnormalities have been found in other autoimmune diseases and if they explain the predisposition to autoimmunity in general, they do not explain the organ/cell-specificity of the autoimmune disease, in particular, that of β cells in diabetes (6).

We therefore hypothesized that abnormalities of the islets of Langerhans, in addition to those in the immune system, are required for the development of the autoimmune reaction at the pancreas level. We have previously reported a transient hyperinsulinemia, which coincided in time with the first pancreas-infiltrating leukocytes (7-9). This hyperinsulinemia was followed by the formation of large islets, or mega-islets (10). These mega-islets were preferentially associated with subsequent infiltrating cells (APC and lymphocytes), indicating a close relationship between these islet abnormalities and the development of the autoimmune reaction (11).

We then addressed the question of the cause of the hyperinsulinemia, which develops from 4 weeks of age onwards, i.e., directly after weaning. The weaning period corresponds to a dietetic shift from maternal milk (rich in fatty acids and poor in carbohydrates) to laboratory chow (poor in fatty acids and rich in carbohydrates) (8,12). Even in normal rodents, this dietetic shift is known to activate β cells that have been at rest until weaning because of the low glucose-containing maternal milk. Since prediabetic NOD mice exhibit β cell hyperactivity immediately after weaning, we wondered whether this was due to a previous glucose stimulation or so-called « glucose-priming effect » (8,13,14). Such a priming effect may occur in NOD fetuses, due to possible abnormalities of glucose homeostasis in NOD mothers.

The measurement of insulin levels in neonatal mice is, however, difficult. To circumvent this problem, we determined whether hyperactivity is present at the transcriptional level in the pancreata of NOD neonates. For this purpose, we developed an *in situ* hybridization technique, that evaluates the rate of preproinsulin II mRNA expression. A cRNA probe complementary to the second intron of insulin II gene was constructed that specifically detects the presence of primary transcripts of this gene (15). The primary transcripts are rapidly processed to mature mRNA and transported into the cytoplasm. For example, *in vitro* studies with corticotrophs demonstrate that the half-life of a primary transcript is only 5-8 min, compared with up 8 hr for the mature form of mRNA (16). The conceptual basis of our assay rests on two facts. First, the levels of intronic sequences, detected by nuclease protection and *in*

situ hybridization reflects more consistently the transcription rate at the nuclear level. Second, in metabolically hyperactive β cells, insulin production is up-regulated at all points of control, including transcriptional activity.

Using this technique we found that 1-day-old NOD neonates exhibit high levels of preproinsulin 2 primary transcript expression compared to age-matched C57BL/6 mice, but the strain difference disappears within the first week of age. Moreover, we tried to prevent this neonatal β cell hyperactivity by treating the NOD mothers with insulin during the last two weeks of gestation. We observed a sexual dimorphism in the response of NOD neonates to insulin treatment: the β cell hyperactivity was down-regulated in female neonates but not in males. NOD*scid* mice lack functional lymphocytes and do not develop insulitis and diabetes (17) but develop a degree of hyperinsulinemia and increased mega-islet formation (8,11). Interestingly, no sexual dimorphism in the response to maternal insulin treatment was observed in NOD*scid* neonates, but in contrast increased β cell hyperactivity was observed.

Results

The distribution of preproinsulin II primary transcript expression is heterogenous

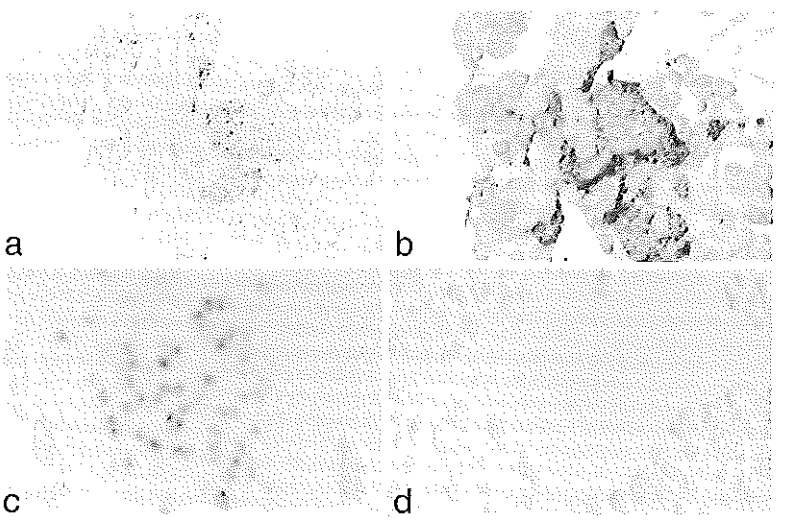

Figure 1. Preproinsulin II primary transcript (ppIns II) expression is restricted to the nucleus of a sub-population of β cells in the islet. *In situ* hybridization for the ppIns II was carried out on paraffin-embedded sections as described in materials and methods. Serial pancreatic sections for (a) *in situ* hybridization and (b) immunostaining for proinsulin (dark grey) and glucagon (black); (c) using an anti-sense probe specific signal for ppIns II were restricted to the nucleus of a subset of β cells; (d) when *in situ* hybridization was carried out with a sense probe, there was a complete absence of staining.

To determine whether β cell hyperactivity is present in NOD neonatal pancreata, an intervening sequence assay was utilized, that exploited the short half-life of primary transcripts in the nucleus as an indirect measure of transcriptional activity. In serial sections stained for the primary transcript or proinsulin and glucagon (Fig. 1a and b, respectively), a specific signal is localized to the β cell area of a subpopulation of islets when compared to proinsulin and glucagon staining. Figure 1c shows a high power view β cell nuclei stained for preproinsulin II primary transcripts. Staining is restricted to the nucleus although not all nuclei are stained in a given islet. Sections hybridized with sense probes have a complete absence of staining (Fig. 1d), similar to sections preincubated with RNase before hybridization (not shown).

NOD neonates have high levels of preproinsulin primary transcript expression

Using a semi-quantitative approach to gauge the level of transcriptional activity, we measured the number of positive nuclei per islet area at 4 different thresholds of staining intensity. Threshold values were arbitrary assigned to encompass the range of staining intensities. It is worth noting here that, in both strains at all ages, the smallest islets exhibit the highest numbers of positive nuclei (results not shown).

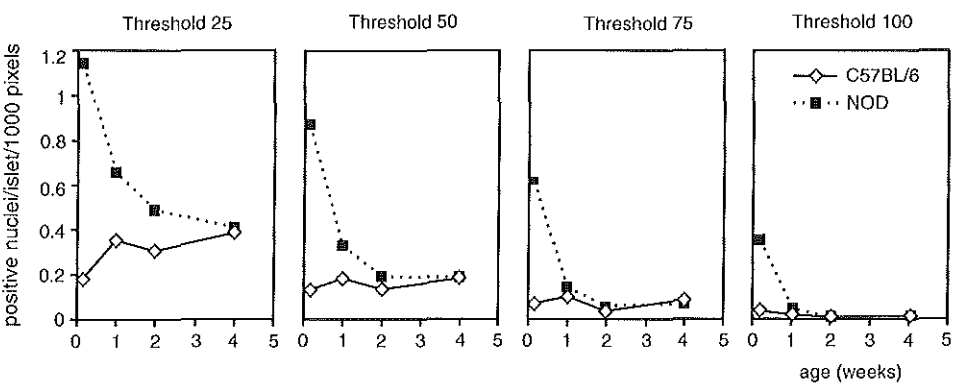

Figure 2. Transcriptional activity is increased in neonatal NOD mice. *In situ* hybridization for the ppIns II was carried out on paraffin-embedded sections as described in materials and methods. A VIDAS image analysis system was used to semi-quantify the number and intensity of stained nuclei. The graph shows the number of nuclei stained per islet area at different arbitrarily assigned thresholds of intensity (25,50,75 and 100) in NOD and C57Bl/6 mice during the early developmental period. Mean ± SEM of 10 mice per age group in each strain.

We also show that 1-day-old NOD neonates have a significantly greater number of stained nuclei when compared to age-matched C57BL/6 mice (Fig. 2). This effect is significant in a three way ANOVA comparing strain, age and threshold ($p<10^{-6}$ in each case). More specifically, *post-hoc* analyses show that the effect is significant between the two strains at

1 day of age and at all threshold levels (p values from 0.02 to 0.00003). However, the difference between strains drops with age such that at 7 days of age there is no significant difference. Thus, NOD neonates have more hyperactive β cells than C57BL/6 neonates, but this difference disappears within the first week of life.

Maternal insulin treatment decreases the level of preproinsulin primary transcript expression in female NOD neonates, but not males

We then investigated whether the β cell hyperactivity observed in NOD neonates is due to possible maternal abnormalities of glucose homeostasis. We therefore treated NOD mares with insulin (1U/100g bw) during the last two weeks of gestation. In this experiment, we analyzed separately female and male neonates for β cell hyperactivity, because of a possible interaction of androgens with insulin action (insulin resistance) (7,18,19). As can be seen from Figure 3, maternal insulin treatment significantly decreases the level of preproinsulin transcriptional activity in female NOD neonates ($p<0.0006$, *post-hoc* analyses), but not in males. This sexual dimorphism in insulin response was observed in two separate experients.

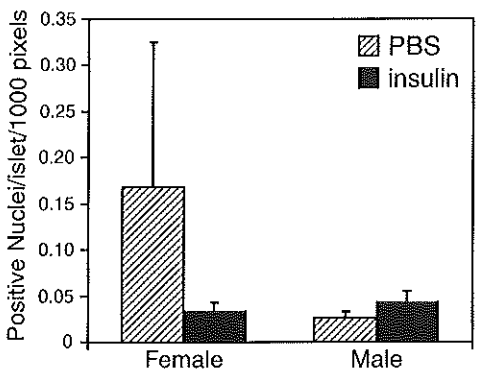

Figure 3. Insulin treatment of NOD mothers diminishes preproinsulin II primary transcript (ppIns II) expression in female NOD neonates only. Results are expressed as in Figure 2. The graph shows the number of nuclei stained per islet area at one threshold of intensity (50), in 1-day-old NOD mice from mothers treated with vehicle or insulin (1U/100g bw). Mean ± SEM of 4 mice per treatment group in each sex.

Maternal insulin treatment increased preproinsulin primary transcript expression in both sexes of NOD*scid* neonates

Insulin is normally not able to cross the placental barrier (20), however, in the case of NOD mice, insulin antibodies are able to transport the hormone into the fetal circulation

(21,22). Under these circumstances, insulin would be able to exert complex effects on the fetuses and not only act by regulating maternal glucose homeostasis. NOD*scid* mice also exhibit a certain degree of hyperinsulininemia after weaning but, because of their lack of functional lymphocytes, do not develop insulitis and diabetes (8). We thought that the use of NOD*scid* would be of help to overcome the problem of the possible presence of insulin antibodies and therefore treated NOD*scid* mothers during the last two weeks of gestation and studied the pancreata of their progeny. As can be seen in Figure 4, the level of preproinsulin transcriptional activity significantly increased in female NOD*scid* neonates ($p<0.03$), while for male NOD neonates only a trend was observed.

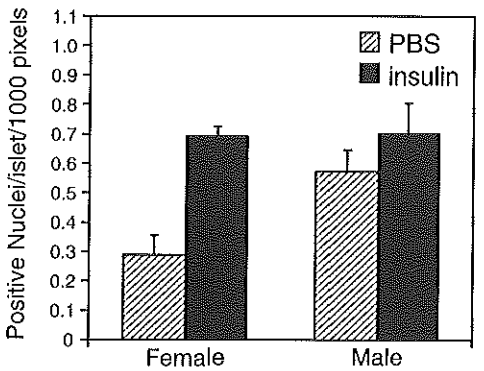

Figure 4. Insulin treatment of NOD*scid* mothers increases preproinsulin II primary transcript expression in both sexes of NOD*scid* neonates. Results are expressed as in Figure 3 at one threshold of intensity (50). Mean ± SEM of 4 mice per treatment group in each sex.

Discussion

We previously demonstrated a transient hyperinsulinemia in prediabetic NOD mice, starting after weaning (7). Here, we analyzed whether this hyperinsulinemia could be the result of an *in utero* « glucose-priming » effect on fetal β cells, because of possible maternal abnormalities of glucose homeostasis (8). Due to the difficulty of measuring circulating insulin in mouse neonates, we set up an *in situ* approach to assess β cell activity in their pancreata.

We showed that NOD neonates exhibit increased β cell activity at birth compared to age-matched C57BL/6 neonates. However, it should be underlined that the distribution of preproinsulin II primary transcripts is highly heterogenous. Indeed, all islets do not express preproinsulin II primary transcripts and not all nuclei in a given islet. Moreover, the difference between the two strains disappears within the first week of age. At this age, pups are fed from birth onwards by low glucose-containing maternal milk that induces a β cell rest which lasts until weaning. We then tried to determine whether the β cell hyperactivity observed in

NOD neonates was linked to maternal abnormalities of glucose homeostasis. Indeed, if most of the NOD mothers are not diabetic at the beginning of gestation, some of them become diabetic during gestation or in the post-partum period.

In order to answer this question, we treated NOD mothers during the last two weeks of gestation at a dose of insulin that lowers glycemia by 50% 2 hr after injection and decreases maternal peri-insulitis and insulitis by about 50% in both cases (results not shown). While levels of β cell activity are similar in female and male NOD neonates from vehicle-treated NOD mothers, maternal insulin treatment exclusively down-regulates it in female neonates. The reason for this sexual dimorphism in β cell reactivity of NOD neonates remains to be determined. We expected a down-regulation of β cell activity in neonates, due to a better control of maternal glucose metabolism. Practically, insulin does not cross the placental barrier unless insulin antibodies are present (20). Indeed, insulin antibodies have been described in NOD mice (21,22). In such a case, insulin might control its own secretion at different levels: at the level of the central nervous system, particularly, during the neonatal period, and at the pancreas level in opposite ways (23-26). The sexual dimorphism could take place at both levels. It is possible also that androgens, produced by male fetuses before birth (27), induce a state of insulin resistance, thereby leading to overworked β cells (8).

We thought that the NOD*scid* model, devoided of functional lymphocytes, but also exhibiting post-weaning hyperinsulinemia (8), can overcome the possible problem of insulin transfer through the placental barrier. In the progeny of insulin treated NOD*scid* mothers, we do not observe a sexual dimorphism in β cell reactivity to insulin, but in contrast an up-regulation in both sexes. An explanation could be the existence of abnormalities of glucose homeostasis in mice with the *scid* mutation, and in particular in NOD*scid*, but to the best of our knowledge this has not been described. However, it is worth noting, here, that nude BALB/c mice, which are deprived of thymus, exhibit several endocrine effects including impaired glucose tolerance at very young age and peripheral insensitivity to insulin (31, 32). In this regard, the nonfasting basal glycemia of NOD*scid* mothers compared to that of NOD was slightly higher 1 day after delivery, whether they have been vehicle- or insulin-treated, in relationship to higher glucagonemia (unpublished results). Therefore, it cannot be excluded that a state of insulin resistance in NOD*scid* mothers may trigger the peculiar β cell response of their female and male progeny.

In conclusion, NOD mice exhibit two waves of β cell hyperactivity: one immediately after birth, which appears to be down-regulated by maternal low-glucose containing milk. The second wave of β cell hyperactivity is observed after weaning, when neonates experience a dietetic shift to high-glucose containing laboratory chow, at a time at which β cells are stimulated even in normal rodents. This second wave of β cell hyperactivity could result from an *in utero* glucose priming effect of fetal β cells, which could be down-regulated, at least in female NOD neonates by maternal insulin treatment during gestation. However, other mechanisms could be involved in the second wave of β cell hyperactivity, such as a stimulatory

effect on insulin secretion of cytokines, for example IL-1, which are produced by the early infiltrating APC in the islet periphery (9,28,29). This second period of β cell hyperactivity might be of importance in the pathogenesis of diabetes. Hyperactive endocrine cells are more prone to autoimmune reactions because of higher levels of autoantigens, adhesion and MHC molecules and because of a higher sensitivity to cytokine-induced damage (6).

Finally, if the maternal environment appears to be of importance in the control of NOD neonatal β cell activity, we can not exclude a genetic component. Moreover, the environment of the fetus (for example, the presence of androgens) may also be at play in controlling β cells. Therefore, the β cell reactivity in NOD mice appears to be a complex event, which occurs very early in life and could depend on genetic and environmental (maternal and fetal) factors. It remains to determine whether the down-regulation of the first wave of the β cell hyperreactivity is able to modify the evolution of the disease.

Materials and methods

Animals and treatments

NOD, NOD*scid and* C57BL/6 mice were bred under specific pathogen-free conditions at the facilities of the Hôpital Necker, Paris, France. The animal facilities and care followed the norms stipulated by the European Community. The incidence of diabetes in the NOD colony is, by 200 days of age, 80% and 40% for females and males, respectively (7). C57BL/6 and NOD mice were sacrificed at 1 day, and 1, 2 and 4 weeks of age in the morning for pancreas sampling. Seven-week-old NOD and NOD*scid* females were mated, with the morning of the appearance of the vaginal plug being taken as the day zero of gestation. Then, they were injected subcutaneously once a day in the late afternoon with 1.0 U human insulin/100 g body weight (Ultratard, NovoNordisk, Boulogne-Billancourt, France) or with the vehicle from days 9 to the last day of gestation (30). Vehicle- or insulin-treated NOD and NOD*scid* mothers were sacrificed 1 day after delivery at the same time as the neonates. Before sacrifice, NOD and NOD*scid* mothers were rapidly bled, as previously described (7), to assess their glycemia using the glucose-oxidase method (Biotrol glucose enzymatic color, Biotrol, Paris, France). Pancreata from NOD mothers were fixed in Bouin's solution (Sigma, Saint-Quentin-Fallavier, France), followed by 10% formalin (Merck, Paris, France) and paraffin-embedded. Sections were cut at 4 µm and stained with hematoxylin eosin (Merck) to assess the degree of insulitis (31).

Tissue preparation

One- and 7-day-old animals were decapitated. Their tissues were immersion-fixed overnight in freshly prepared 4% paraformaldehyde. Animals over 14 days old were anaesthetized with avertin (Sigma), flushed with PBS containing heparin (10 U/ml) and 0.5% w/v $NaNO_2$ and perfused with 4% paraformaldehyde. Tissues were harvested and placed in fixative for a further 2 hours. After fixation tissues were washed in 70% alcohol and embedded in paraffin using routine procedures. Sections of 4-5 μm were cut and placed on 3-aminopropyltriethoxysilane (Sigma) coated slides and stored at RT.

Construction of the murine Ins II IVS probe

A cRNA probe specific for the primary transcript of preproinsulin II was generated based on a previous method (16). PCR primers were designed that spanned 354 bp of the second intron (representing 72% of its sequence). The amplification product was generated by standard RT-PCR amplification from total pancreatic extracts as described previously (32) and purified on a 6% polyacrylamide gel. The resulting cDNA fragment was ligated into a pGEM-T Easy vector (Promega, Charbonnières, France) according to manufactures' instructions. Plasmid DNA was prepared from recombinant clones, linerized, proteinase K digested and phenol/chloroform extracted. Digoxigenin (DIG)-labeled sense and anti-sense probes were synthesised from appropriately linerized DNA using the *in vitro* translation system in the presence of DIG-11-UTP, according to manufactures' instructions (Boehringer Mannheim, Mannheim, Germany) with SP6 or T7 RNA polymerase. The DNA template was digested by DNase I and the RNA probe NH_4Cl precipitated and purified by spin column chromatography (Clontech, Palo Alto, Ca). Probes were aliquoted and stored at -70ŸC.

In situ hybridization

Tissue sections were deparaffinized, cleared and rehydrated then subjected to proteinase K digestion (100 μg/ml) for 30 min at 25ŸC. The reaction was stopped with 0.2% glycine and sections were re-fixed in 4% paraformaldehyde for 10 min. Sections were then rinsed in H_2O, dehydrated and air-dried. Sections were then covered with 30-40 μl of hybridization buffer (HB) pH 6.8, containing 100 ng/ml of DIG-labeled antisense or sense probe, 300 mM NaCl, 10 mM Na_2HPO_4, 10 mM Tris-Cl, 50 mM EDTA, 50% deionised formamide, 5% dextran sulphate, 100 μg/ml tRNA, 100 μg/ml sonicated salmon DNA. Sections were incubated overnight at 50ŸC in covered chambers humidified with HB without probe and 50% formamide to maintain vapour pressure. After hybridization sections were incubated for 4 hrs in 2 changes of HB without probe and 50% formamide at 50ŸC to remove probe, washed 3x in RNase buffer (600 mM NaCl, 10 mM Tris-HCl pH 7.2, 50 mM EDTA) and

incubated with 30 μg/ml RNase for 60 min at 37YC. Slides were washed in PBS and incubated in 2 changes of 2x SSC at 63YC for 60 min. Slides were then washed in Tris-buffered saline containing 0.05% Tween 20 (TNT) and incubated overnight at 4YC with anti-DIG F(ab) fragments (Boehringer) at a dilution of 1/750. At the end of the incubation slides were washed first in TNT, then in alkaline Tris-buffer saline containing 50 mM $MgCl_2$ pH 9.5. Slides were then placed in slide mailers containing 4.5 μg/ml nitroblue tetrazolium (NBT) (Boehringer) and 3.5 μg/ml 5-Bromo-4-chloro-3-indolyl-phosphate Na_2 (BCIP) (Boehringer) and left upright at RT for 4-6 hr. Positive control sections were checked for staining intensity then sections were washed in buffer containing 10 mM EDTA, dehydrated and mounted in Eukitt.

Immunohistochemistry for proinsulin and glucagon

Double labeling was performed on 4 μm sections cut from paraffin-embedded tissues. After dewaxing and rehydrating, the tissues were washed in Tris-saline buffer (0.1M Tris-HCl, pH 7.5, 0.15M NaCl, 0.05% Tween 20). Sections were incubated with optimally diluted guinea pig anti-insulin serum (Linco Research Inc., St. Charles, MO) and rabbit anti-glucagon serum (Chemicon International Inc, Temecula, CA) overnight at 4YC. Slides were thoroughly washed in Tris-saline buffer and incubated first with swine anti-rabbit IgG conjugated with alkaline phosphatase (DAKO, Trappes, France) for 30 min at RT, rinsed and then incubated with rabbit anti-guinea pig IgG conjugated with horseradish peroxidase (DAKO, Trappes, France) for a further 30 min. Sections were rinsed first in Tris-saline buffer (pH 7.5), then briefly in alkaline Tris-saline buffer (pH 9.5 containing 50 mM $MgCl_2$). Immunoreactive glucagon was revealed using a substrate consisting of 0.34 mg/ml NBT and 0.18 mg/ml BCIP. Slides were protected from light and periodically checked for staining intensity. The reaction was stopped by returning sections to neutral pH in Tris-saline buffer containing 10 mM EDTA. Slides were washed thoroughly for at least 30 min, then reacted with the peroxidase chromogen diaminobenzidine (Sigma) in PBS with 0.02% H_2O_2 for 1-2 min. Slides were finally rinsed in water counterstained with methyl green (Fluka, Buchs, Switzerland), dehydrated through alcohol, cleared and mounted.

Image analysis

We performed a semi-quantitative analysis of preproinsulin II expression via a VIDAS-RT image analysis system (Kontron Elektronik GmbH/ Carl Zeiss, Weesp, The Netherlands). Measurements were done at a magnification of 20x. Islets were identified under phase contrast and circled. Positively stained nuclei inside individual islets were enumerated at four thresholds of staining intensity. To correct for the influence of islet size, data are expressed for each islet as the number of positive nuclei per 1000 pixels. It should be realized

that the intensity of staining differs between different experiments while the threshold levels are set. Therefore, the data are relative and can only be compared within and not between experiments.

Statistical analysis

Values are mean ± SEM of 4 to 10 neonates per age group in each strain depending on the experiment. For each animal, 3 noncontiguous sections were analyzed. Data were analyzed with Statistica software (Statsoft, Tulsa, OK). Possible effects of strain, age and treatment were analyzed using ANOVA for each variable investigated. *Post-hoc* analysis, using the Tukeys HSD method was performed when effects and interactions were significant ($p<0.05$), as assessed by ANOVA.

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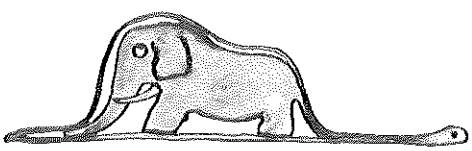
Chapter 3.4

NOD mouse dendritic cells aberrantly stimulate islet insulin release in vitro



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Summary

The pathogenic process causing autoimmune diabetes in the nonobese diabetic (NOD) mouse starts around 4 weeks of age with an accumulation of antigen-presenting cells (APC), especially $CD11c^+$ dendritic cells (DC), around the pancreatic islets of Langerhans. Concomitantly, a hyperinsulinemia is observed, which may either be cause or consequence of the initial APC infiltration. To determine whether infiltrating DC can cause such an islet abnormality, we have performed experiments in which isolated DC are co-cultured with isolated islets of Langerhans.

We first show that islets isolated freshly from 6-8 week old prediabetic NOD mice have higher insulin and glucagon contents than islets of C57BL/6 control mice. Moreover, as is the case *in vivo*, islets from prediabetic NOD mice do secrete more insulin *in vitro* than age-matched C57BL/6 mice. Only in NOD cocultures, the addition of DC results in an increased secretion of insulin; glucagon secretion is not enhanced under these conditions.

These findings indicate that infiltrating DC may play a role in the islet hyperactivity, observed both *in vitro* and *in vivo*, and which might sensitize the NOD islet for autoimmune attack.

Introduction

The nonobese diabetic (NOD) mouse is a widely used model for type 1 (insulin-dependent) diabetes mellitus (type 1 diabetes) (1,2). In this model, autoimmunity towards pancreatic islets of Langerhans starts with an accumulation of antigen-presenting cells (APC) around the islets of Langerhans. These APC include ER-MP23$^+$ macrophages (Mϕ) and CD11c$^+$ dendritic cells (DC) (3,4). Subsequently, lymphocytes accumulate at the periphery of the islets (peri-insulitis). APC and lymphocytes start to infiltrate the islets (insulitis) only after this peri-islet lymphocytic infiltration. This process, which is associated with the infiltration of BM8$^+$ Mϕ into the islets, ultimately results in the destruction of the insulin-producing β cells and the generation of symptoms of hyperglycemia (3,4).

Little is known about the initial trigger for the early accumulation of APC around the islets. In this respect, the islet itself may play an important role in initiating its own destruction (5). Several islet abnormalities have been shown in the NOD mouse that coincide in time with the first signs of APC infiltration, such as a transient hyperinsulinemia (6) and hyperglucagonemia (Pelegri et al., submitted) and the formation of an increased frequency of large islets (7).

These pre-insulitic islet abnormalities may play a role in the breakdown of immunological tolerance towards the insulin-producing β cells by attracting DC (5). These DC may be attracted because of the aberrant endocrine function, since DC have been shown to regulate endocrine cell function and growth in several endocrine organs (8). For example, relatively large numbers can be found in the anterior pituitary, where DC form a subgroup of the network of folliculo-stellate cells (9) that regulates the hormonal responses of neighbouring endocrine cells (10,11). In the ovary and the testis, APC play a role in the regulation of ovulation (12) and in growth and steroid production of granulosa, theca, luteal and Leydig cells (8,13,14). DC are also present in low numbers in the normal thyroid (15,16) and modulate the growth of thyrocytes (17). Cytokines, such as IL-1, IL-6 and TNF, play a major role in these modulating actions (8). For pancreatic islets, however, a similar regulating role of DC has not been shown.

Thus, a physiological response may explain the specific attraction of DC towards dysfunctioning islets. However, the islet abnormalities coincided in time with the influx of APC, leading to the possibility that the abnormalities are the consequence and not the cause of infiltrating APC. Thus, the exact relationship between early islet abnormalities and early infiltrating APC in the NOD mouse model of type 1 diabetes is unclear at present.

In this study, we address the question whether DC are able to influence the activity of pancreatic islet cells. For this purpose, we have isolated islets and cultured them in the pres-

ence of isolated DC to determine their effects on islet activity, as assessed by insulin and glucagon secretions and contents. Furthermore, we compare the effect of DC on islets isolated from normal C57BL/6 and autoimmune-prone NOD mice.

Results

NOD islets contain and secrete more insulin in vitro than C57BL/6 islets

We previously showed a transient hyperinsulinemia in prediabetic 4- to 8-week -old NOD mice, when compared to mice from various control strains, in particular the C57BL/6. This transient hyperinsulinemia was rapidly followed by an increased frequency of so-called mega-islets in NOD mice. We therefore assessed whether a similar NOD/C57BL/6 difference in insulin secretion and/or content could also be observed *in vitro*. Indeed, the insulin contents of islets isolated from prediabetic (8-week-old) NOD mice measured at the start of the incubation (immediately *ex vivo*) were significantly higher than in C57BL/6 mice (14797±3332 versus 6192±815 µU/well, $p<0.004$). Moreover, islets from prediabetic (8-week-old) NOD mice secreted significantly more insulin in a 24h-culture than those from C57BL/6 mice at the stimulating concentration of glucose used (11 mM) ($p<0.002$) (Fig. 1). This is accompanied by a significant decrease of insulin contents after 24 h incubation only in the NOD strain ($p<0.0006$, Fig. 1). The enhanced insulin secretion by NOD islets *in vitro* is reminiscent of the hyperinsulinemia which can be found at the same age in NOD mice *in vivo*.

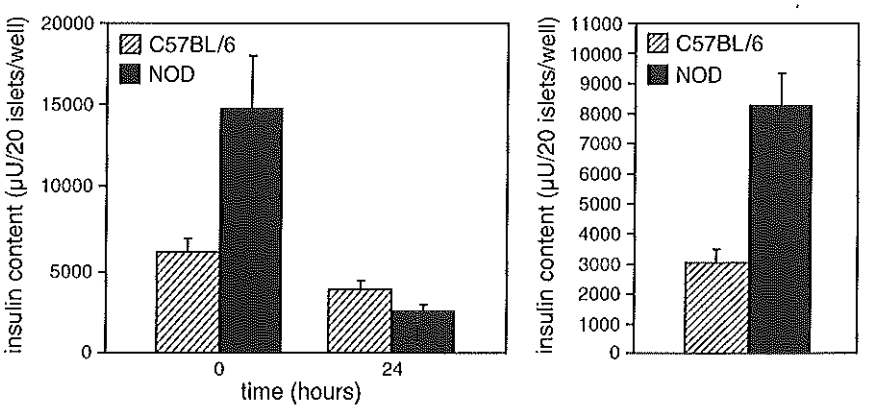

Figure 1: Insulin content as a function of culture time in islets from C57BL/6 and NOD mice (left figure) and insulin secretion during 24h of culture (right figure). NOD mice have significantly higher insulin contents than C57BL/6 mice directly after isolation. After 24h of culture, insulin content in NOD and C57BL/6 mice are comparable, due to a significantly higher insulin secretion of NOD islets.

Thus, the behaviour of the islets in these culture conditions reflects their behavior *in vivo*, since NOD females have higher blood insulin responses than C57BL/6 females to intraperitoneally glucose administration (6).

NOD islets have higher glucagon contents than C57BL/6 islets

Since insulin- and glucagon-secretions are tightly co-regulated, we also investigated islet glucagon contents and secretion. As already observed for insulin contents, glucagon contents were also significantly higher in NOD islets than in C57BL/6 ones at the start of the incubation (19540±3970 pg/well for NOD and 10771±1737 pg/well for C57BL/6, $p<0.03$) (Fig. 2). We previously demonstrated significantly higher blood glucagon levels in prediabetic NOD mice *in vivo* as compared to control C57BL/6 mice (Pelegri et al., submitted). Indeed, after a 24 h *in vitro* incubation at high glucose concentrations, glucagon secretion appeared to be higher in islets from NOD than from C57BL/6 mice, although this difference was not statistically significant (Fig. 2). However, islet glucagon contents after 24 h of culture were significantly decreased in NOD islets, but not in C57BL/6 islets ($p<0.02$, Fig. 2).

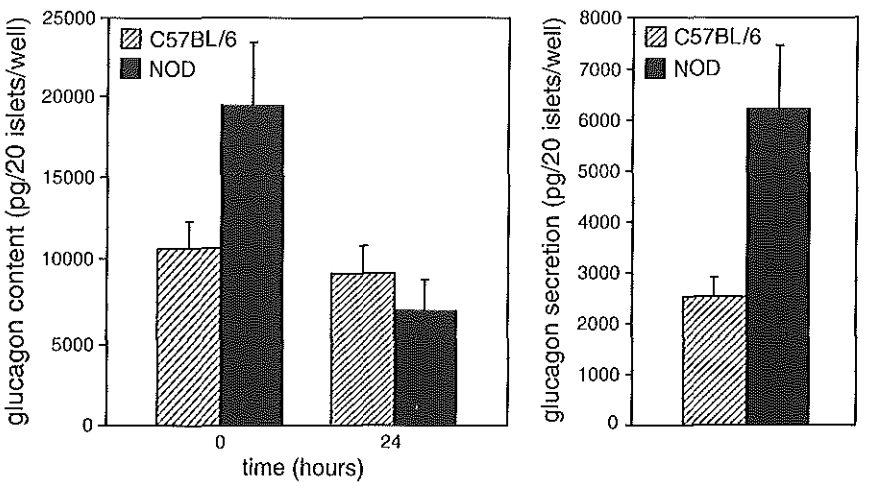

Figure 2: Glucagon content in islets from C57BL/6 and NOD mice directly after isolation and after 24 hours of culture (left figure) and glucagon secretion during 24h of culture (right figure). After isolation, NOD mouse islets have significantly higher glucagon contents than C57BL/6 islets . After 24h of culture, glucagon content in NOD and C57BL/6 mice are comparable, related to a higher glucagon secretion of NOD islets.

NOD DC stimulate islet insulin release in vitro, whereas C57BL/6 DC have no effect

To determine whether infiltrating DC could cause early islet abnormalities, co-culture experiments were performed in which three different DC concentrations were added to islets

of the same strain. In C57BL/6 mice, the addition of DC did not significantly affect *in vitro* islet insulin release, regardless of the DC concentration used (Fig. 3). However, the addition of NOD DC at the two lowest concentrations to NOD islets significantly stimulated islet insulin release ($p<0.02$) (Fig. 3).

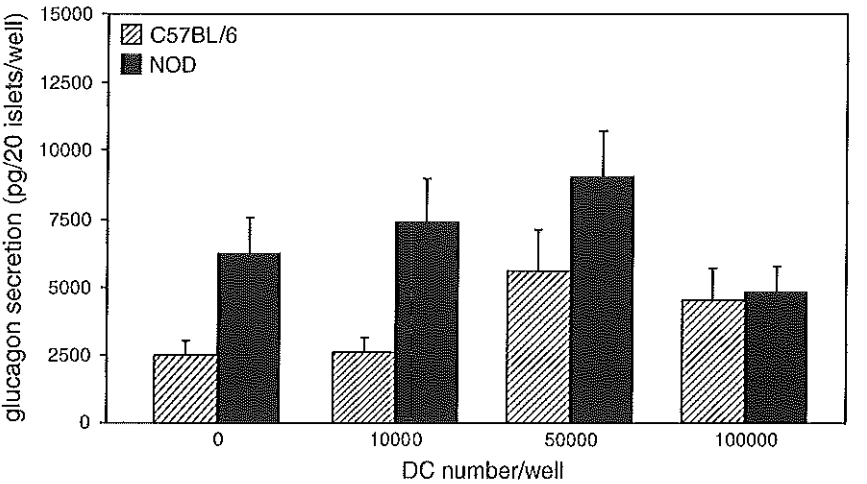

Figure 3: Insulin secretion in co-cultures of C57BL/6 and NOD islets with different numbers of syngeneic DC. Lower concentrations of NOD DC, but not C57BL/6 DC, stimulate islet insulin release in vitro.

The addition of DC from either NOD or C57BL/6 to their syngeneic islets did in no case significantly influence islet glucagon release (Fig. 4).

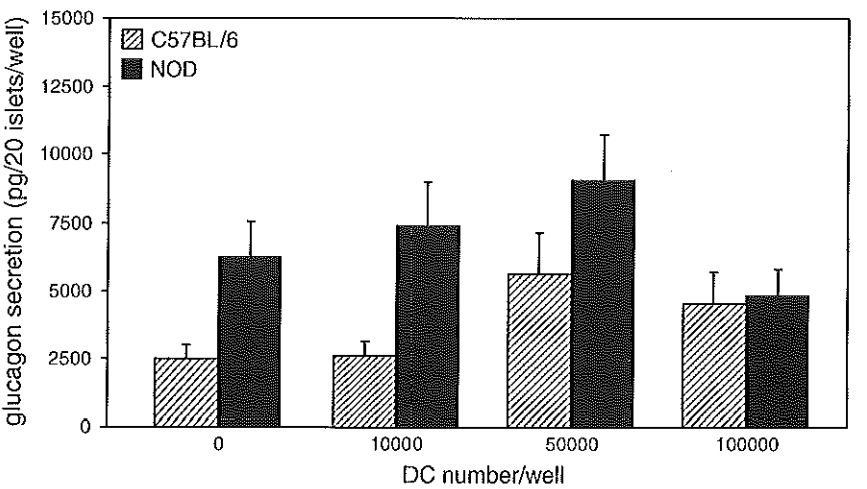

Figure 4: Glucagon secretion in co-cultures of C57BL/6 and NOD islets with different numbers of syngeneic DC. DC from both NOD and C57BL/6 mice do not significantly influence islet glucagon release *in vitro*.

It is worth noting that morphological changes were observed only in the C57BL/6 DC-islet-coculture after 24h of incubation, but not in the NOD coculture. In C57BL/6 cultures, DC appeared to be attracted to the islets and intermingled with a few fibroblasts around the islets. Moreover, the appearance of small, duct-like structures was observed in these C57BL/6 DC-islets cocultures.

In summary, in these experiments NOD mouse DC were able to stimulate islet insulin secretion, but not glucagon secretion. DC from control mice did not affect islet insulin, nor glucagon release.

Discussion

The presence of leukocytes such as DC in normal endocrine glands and their role in the physiological control of endocrine function raises questions about their possible patho-physiological role in endocrine autoimmunity. Suggestive of such a pathogenic role is the observation that the accumulation of DC in the endocrine target organ is usually the first sign of self-reactivity. This is observed at the thyroid and the islet level in spontaneous animal models of endocrine autoimmunity, such as the BB-rat (18) and the NOD mouse (3). The presence of DC has also been described in human endocrine autoimmunity, in the thyroid (16) and in pancreatic islets (19). In NOD mice, the presence of $CD11c^+$ DC coincides in time with the appearance of early islet abnormalities. Therefore, we approached the question whether early islet abnormalities are cause or consequence of DC infiltration. In order to answer this question, we performed cocultures of isolated islets of Langerhans and DC of NOD and control C57BL/6 mice.

We first found that NOD islets isolated from 6-8 weeks old mice contain more insulin and glucagon than islets from C57BL/6 mice. Furthermore, NOD islets secrete in particular more insulin. These *in vitro* findings clearly parallel our previous results *in vivo*, since from 4-8 weeks of age, NOD mice show an islet hyperactivity, as evidenced by elevated basal blood insulin levels and increased insulin response to intra-peritoneal glucose administration (6). There is also, but to a lesser extent, an increase of blood glucagon levels in NOD mice at 4-8 weeks of age (Pelegri et al., submitted).

In vivo, islet hyperactivity is followed in time by an islet hypertrophy, as evidenced by an increased number of very large islets, that start to develop between 5 and 10 weeks of age. Both APC and lymphocytes are preferentially situated near these mega-islets (Rosmalen et al., submitted). This association between infiltrating cells and mega-islets is especially strong for the $CD11c^+$ DC. This association may be explained by two non-mutually exclusive possibilities: mega-islets, by virtue of an enhanced release of antigen and other factors, may specifically attract these infiltrating cells; alternatively, infiltrating cells may primarily accu-

mulate in the islet area for an as yet unknown reason, and cause the islet hyperactivity observed *in vivo* and *in vitro*, followed by islet hypertrophy.

The current coculture experiments of NOD DC and islets suggest that the second possibility may play a role. We show that NOD DC stimulate islet insulin release *in vitro*, whereas DC from C57BL/6 mice have no such effect when added to C57BL/6 islets. Thus, the accumulation of DC around the islets can contribute to the observed endocrine islet abnormalities. It is worth noting here that a similar mechanism seems to take place in the BB rat thyroiditis. In this model, the initial influx of DC coincides in time with abnormalities in thyroid T_3 production and an enhanced proliferation of thyrocytes (17).

From our current data we can not conclude whether the enhanced insulin release in cocultures from NOD DC and islets is due to aberrations in the DC or in the islets. Preliminary experiments indicate that C57BL/6 DC are not able to affect insulin release of NOD islets significantly. These data suggest that the enhanced insulin release, as observed in cocultures of NOD islets and NOD DC, can be attributed to an aberrant DC function in NOD mice. These experiments however await confirmation.

Thus, NOD DC, but not C57BL/6 DC seem to stimulate syngeneic islet function. It can not be excluded, however, that contaminating cells in splenic DC isolates, such as lymphocytes, contribute to the observed insulin release of NOD islets cocultured with DC. Since islets cocultured with DC do not show any signs of destruction, it is unlikely that direct lymphocyte- (or DC-) mediated islet cell-cytotoxicity as the cause for the enhanced insulin levels in the culture medium. Finally, a significant stimulating DC effect was only observed at the two lowest DC concentrations, suggesting that too many DC, as added in the highest concentration, were able to inhibit a fully stimulated β cell function. This can be explained by the fact that cytokines, such as the APC-product IL-1, may stimulate or inhibit islet function depending on their concentration (20,21). Thus, cells that secrete such cytokines may exert opposite effects on the islets, depending on their cell number. Therefore, the enhanced insulin release that is observed in NOD cocultures is not likely to be due to cytotoxic effects of contaminating cells.

Finally, it should be noted that in both spontaneous animal models (22,23) and human autoimmune diabetes (24,25), APC have been shown to be abnormal in their immune regulatory function. These defects in immune regulation are thought to be important in the loss of tolerance towards self-components. However, as DC have, besides antigen presentation, an additional function in endocrine regulation, it is tempting to speculate that this function may also be abnormal. In line with this, it has been shown that DC of autoimmune-prone BB rats are defective in regulating the growth of thyrocytes (17).

In conclusion, DC appear to play an important role in the communication between the immune system and the pancreas in the pathology of type 1 diabetes. This makes them interesting candidates for a role in causing endocrine autoimmunity.

Materials and methods

Animals

NOD and C57BL/6 female mice were bred under specific pathogen-free conditions at the facilities of the Hôpital Necker, Paris, France. The animal facilities and care followed the norms stipulated by the European Community. The incidence of diabetes in the NOD colony is, by 200 days of age, 80% for females and 40% for males. For coculture experiments, DC and islets were isolated from 6 to 8-week-old C57BL/6 control and prediabetic NOD mice.

Isolation of islets

Ten mice were sacrificed by cervical dislocation for a given islet isolation, their pancreases were removed and cut into small pieces in PBS supplemented with 5% fetal calf serum (Biological Industries, Kibbutz Beit Haemek, Israel), 100U/ml penicillin / 100µg/ml streptomycin (Gibco BRL, Life Technologies, Cergy Pontoise, France) and 0.05% glucose (Laboratoires Meram, Rhône Poulenc, Melun, France) (PBS/5% FCS). These pieces were incubated with thorough shaking with 40 mg collagenase P (Boehringer Mannheim, Mannheim, Germany) in 8 ml PBS supplemented with 15% FCS, penicillin/streptomycin and glucose, for 6 minutes at 37°C. When pieces had visibly dispersed, the digestion was stopped by the addition of ice-cooled PBS/ 5% FCS and the tissue digest was washed 3 times in ice-cooled PBS/5% FCS. Islets were purified as follows on a discontinuous Euro-Ficoll density gradient, obtained by adding 34%, 23%, 20% and 11% (w/v) Ficoll 400 (Sigma, St Quentin Fallavier, France) to Eurocollins solution (Fresenius France, Division Biosedra Pharma, Louviers, France). After removal of the last washing supernatant, 3 ml of 34% Euro-Ficoll were mixed with the pelleted tissue digest. To this tissue layer, 3 ml of 23% Euro-Ficoll, 2 ml of 20% Euro-Ficoll and 2 ml of 11% Euro-Ficoll were gently added. Purified islets were collected from the upper two interfaces and washed twice in PBS/5% FCS. Islets were kept on ice in MEM glutamax (Gibco) supplemented with 10% FCS, 1% sodium-pyruvate (Gibco), 1% non-essential amino acids (Gibco), 1% penicillin-streptomycin and aprotinin (2000 KIU/ml) (Bayer Pharma, Puteaux, France). MEM glutamax (5.5 mM glucose) was also supplemented with extra glucose as the culture medium throughout the study contained 11.1 mM glucose to mimick high glucose levels *in vivo*.

The purity of islets was checked with a dithizone staining. For this staining, 1 ml DMSO (Sigma) was added to 30 mg dithizone (Sigma). PBS was added to a final volume of 10 ml, and the solution was filtered through a 0.2 µm mesh. 200 µl of DMEM with purified islets was added to 150 µl of the dithizone solution in a petri-dish with grid. Islets were counted and checked for purity.

Isolation of DC

Three mice were sacrificed by cervical dislocation, their spleens were removed, pooled and cut into small pieces. Cells were incubated for 60 minutes at 37 °C with 130 U/ml collagenase III (Worthington Biochemical, Freehold, NY) and 0.1mg/ml DNAseI (from bovine pancreas, grade II; Boehringer Mannheim GmbH, Germany) in RPMI (Gibco) supplemented with 25 mM Hepes and 1% penicillin/streptomycin. The tissue was teased through a 40 mm filter and washed with RPMI supplemented with 25 mM Hepes, 1% penicillin/streptomycin and 10% FCS (RPMI/FCS). All washing steps included centrifugation for 5 minutes at 400g. The cell pellet was resuspended in erythrocyte shock buffer (NH_4Cl 9.84 g/l, $KHCO_3$ 1 g/l, EDTA 0.1 mM) and incubated for 10 minutes on ice. Cells were washed again with RPMI/FCS, resuspended in RPMI/FCS at 10^7 cells/ml and cultured overnight at 37°C and 5% CO_2. Non-adherent cells were collected and resuspended in RPMI/FCS at a concentration of 1-2.10^7 cells/ml. Two ml of the cell suspension was layered on top of 2 ml 14% Nycodenz (Nycomed Pharma As, Oslo, Norway) in RPMI/FCS. This gradient was centrifugated for 20 min at 530 x g and the interphase was collected. The interphase was mixed with RPMI/FCS 1:1 and this mix was centrifuged for 10 min at 400 x g. Finally, the cells were washed with RPMI/FCS. The purity of the DC was checked by FACS-staining using N418-FITC identifying $CD11c^+$ DC, and the resulting cell suspension was about 70% $CD11c^+$. Contaminating cells were primarily B lymphocytes.

Islet-DC cocultures and insulin and glucagon determination

Islets and DC were used immediately after isolation and counting. Incubations were performed in 24-well plates (Falcon, Polylabo, Strasbourg, France). 20 islets were distributed per well with or without varying numbers of DC (1.10^4, 5.10^4, 1.10^5). The total volume of incubation was 500 µl/well.

Part of the wells without DC were used immediately after the distribution of the islets in order to determine total insulin and glucagon contents at the beginning of the culture. Supernatants and islets were collected and sonicated for 10s in 500 µl acid-ethanol (1.5% (v/v) 1N HCl in 75% ethanol). After overnight extraction at 4°C, the samples were stored at -20°C until the insulin or glucagon concentration was determined by radioimmunoassay.

The wells containing islets with or without DC were cultured for 24h at 37°C in an atmosphere of 5% CO_2 in humidified air. The incubation media of islets alone (controls) or cocultured with DC were collected and frozen at -20°C prior to RIA for insulin or glucagon. For the measurement of the islet hormone contents after 24h incubation, islets were collected in 500 µl of acid-ethanol as described above. The extracts were stored at -20°C before RIA. Insulin and glucagon concentrations in medium were determined using standard RIA kits (SB-INSULIN CT, CIS Biointernational, Gif-sur-Yvette, France for insulin; Biodata, Pharmacia, Saint-Quentin-en-Yvelines, France for glucagon).

Statistical analysis

All data are expressed as mean ± SEM (n=8/group). Statistical significance for differences in insulin and glucagon levels was determined by means of a 2-way ANOVA. *Post-hoc* analysis, using the Newman-Keuls test, was performed when ANOVA revealed that effects and interactions were significant ($p<0.05$).

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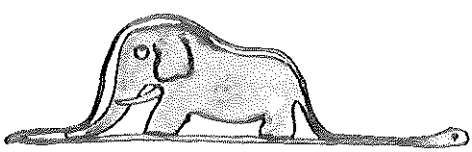
Chapter 3.5

Islet α cell disturbances from birth onwards in mice with the nonobese diabetic genetic background



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Summary

Although several defects in immune function have been observed in prediabetic and diabetic animals and humans, these do not explain the organ specificity of the autoimmune reaction. In the search for prediabetic islet abnormalities, we previously found transient hyperinsulinemia in nonobese diabetic (NOD) mice. To determine whether hyperglucagonemia might play a role in triggering this hyperinsulinemia, various α cell parameters were compared in NOD, lymphocyte-deficient NOD*scid* and control C57BL/6 females from birth onwards.

Before weaning, blood and pancreatic glucagon levels were significantly lower in NOD and NOD*scid* compared to C57BL/6 mice. In contrast, after weaning, glucagonemia was higher in NOD and NOD*scid* than in C57BL/6 mice. To analyze in situ α cell behavior, glucagon immunohistochemistry was combined with image analysis, so as to calculate the number of islets of a given size and the percentages of islet glucagon-positive areas as a function of islet size. From birth onwards, the percentages of glucagon-positive areas were higher in NOD pancreata than C57BL/6. This difference was principally observed at the level of small-sized islets in NOD mice, while only a trend was found in NOD*scid*. Moreover, NOD and NOD*scid* mice, particularly at 1 day of age, had twice as many very small islets as C57BL/6 mice.

These data suggest that, before weaning, NOD, and to some extent NOD*scid*, pancreata contain more immature islets (as reflected by their small size and high percentages of glucagon-positive areas, concomitant with lower glucagon secretion and storage) than C57BL/6 pancreata. This islet immaturity might play a role in the initiation of islet autoimmunity.

Introduction

Despite extensive genetic and immunological research, the complex etiology and pathogenesis of type 1 (insulin-dependent) diabetes mellitus (type 1 diabetes) remain unresolved (1). In addition to investigations in humans, two spontaneous animal models of the disease have been helpful in these studies, namely the bio-breeding rat and the nonobese diabetic (NOD) mouse (2,3). In all cases, type 1 diabetes is a polygenic disease, with multiple defects in various immune-cell types (2,3). However, these immune dysfunctions cannot explain the organ specificity of the autoimmune process. It has been suggested that abnormalities in the islet itself may play an important role in the breakdown of tolerance towards islet β cells (4).

In our search for such prediabetic islet anomalies, we demonstrated a rather surprising but transient β cell hyperactivity in prediabetic NOD mice (5). Indeed, we observed higher insulin levels and lower glycemia values in 4- to 8-week-old NOD mice, particularly females, as compared to mice from various control strains (4). Hyperinsulinemia was also shared to some extent by the NOD*scid* mouse, which lacks functional lymphocytes and therefore does not develop lymphocytic insulitis or diabetes (6). Hyperinsulinemia in NOD and NOD*scid* mice appears just after the change of diet at weaning, which normally takes place at about 3 weeks of age in all mice. It is also worth noting that this is the time at which the primary infiltrating immune cells (dendritic cells and macrophages) begin to reach the periphery of the islets of Langerhans in both NOD and NOD*scid* mice (4,7,8). Hyperactive islets may be essential for the progression of the disease in NOD mice, since islets in the activated state are known to express elevated levels of cell-adhesion ligands, major histocompatibility complex molecules and autoantigens, which may heighten the sensitivity of β cells to the cytotoxic effects of cytokines (9).

The cause of the transient β cell hyperactivity observed in NOD and NOD*scid* mice is not known. Experimental data suggest that hyperglucagonemia, among other factors, might be involved in NOD β cell hyperactivity. Hyperglucagonemia stimulates insulin secretion in vitro by pancreata from normal animals and those with spontaneous type 2 diabetes (10-12). Indeed, hyperglucagonemia has been described in prediabetic (2-month-old) NOD mice, compared to their ICR littermate controls (13). Therefore, we hypothesized that hyperglucagonemia was involved in the transient NOD β cell hyperactivity. To investigate in greater depth the role of glucagon in β cell hyperactivity, we determined α cell presence and activity in mice with the NOD genetic background (NOD and NOD*scid*) as compared to control mice (C57BL/6). This assessment was done from birth to 10 weeks of age, because the autoimmune reaction involving antigen-presenting cells and lymphocytes progresses during this time frame at the NOD pancreas level, but clinical onset of diabetes is not yet observed. NOD*scid* mice, which also show transient hyperinsulinemia but no insulitis or diabetes due

to the absence of functional lymphocytes, were used to distinguish between the early endocrine abnormalities that exist on a NOD genetic background and therefore precede the lymphocytic infiltration, and those that are induced by infiltrating lymphocytes as observed in NOD mice.

Results

NOD and NODscid blood glucagon levels are lower before and higher after weaning compared to C57BL/6 mice

To investigate whether hyperglucagonemia could play a role in the previously observed β cell hyperactivity in NOD mice, we determined nonfasting blood glucagon levels in NOD, NOD*scid* and C57BL/6 mice. Figure 1 shows circulating glucagon levels as a function of age in the three strains.

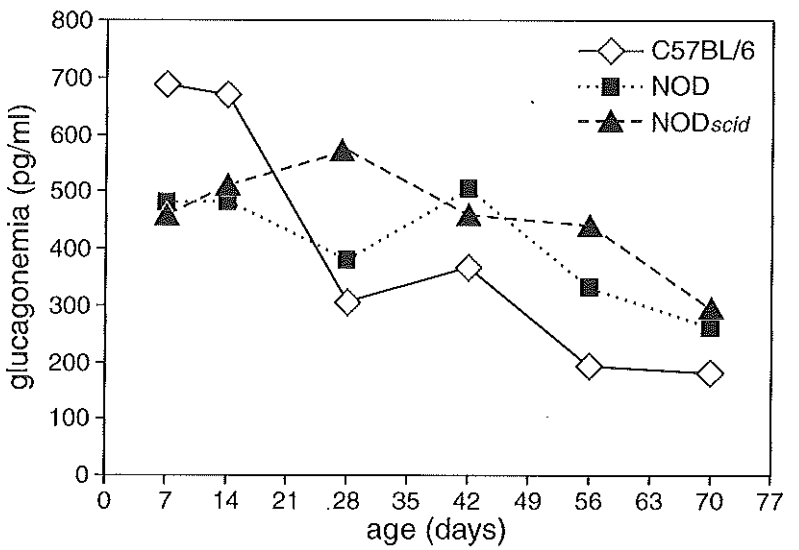

Figure 1: Basal nonfasting blood glucagon levels in C57BL/6, NOD and NOD*scid* females as a function of age. Values are expressed as means ± SEM (n=15/age/strain). Individually, strain and age significantly affected nonfasting blood glucagon levels ($p<0.01$ and $p<10^{-6}$, respectively) and there was also a significant strain x age interaction ($p<10^{-6}$).

While blood glucagon levels decreased with age in all strains, the patterns of decrease differed between the control strain (C57BL/6) and the strains with the NOD genetic background. In C57BL/6 mice, as was already described for normal rodents (14-18), glucagon levels were relatively high before weaning and declined significantly thereafter. In particular,

glucagon levels in C57BL/6 mice at 7 or 14 days of age were significantly higher than those found at later time points ($p<10^{-4}$); the most significant decline was observed between days 14 and 28 of age in close relationship to weaning that occurs at 21 days of age. In NOD and NOD*scid* mice, a decrease of blood glucagon concentrations was also observed with age, but it was delayed and less marked compared to C57BL/6.

These different kinetics of blood glucagon concentrations in NOD and NOD*scid* mice were the reflection of differences in circulating glucagon levels at a given age. Before weaning, on days 7 and 14, nonfasting blood glucagon concentrations were significantly higher in C57BL/6 than in NOD ($p<0.01$) and NOD*scid* ($p<0.05$) mice. By contrast, after weaning, glucagon concentrations in NOD and NOD*scid* were constantly higher than those in C57BL/6 mice, with differences reaching significance at some time points, in particular on days 28 and 56 ($p<0.05$ and $p<10^{-4}$, respectively).

In summary, blood glucagon levels at different ages differ between control mice and mice with the NOD genetic background. Before weaning, NOD and NOD*scid* mice have lower circulating glucagon levels and, after weaning, higher glucagon levels than control C57BL/6 mice.

NOD and NOD*scid* mice have low glucagon pancreatic contents before weaning compared to C57BL/6 mice

The lower blood glucagon levels in NOD and NOD*scid* mice before weaning might be due to either low synthesis, low secretion or enhanced glucagon turnover. In an attempt to answer this question, we determined the amount of pancreatic stored glucagon in NOD, NOD*scid* and C57BL/6 mice.

Because of the marked growth of the pancreas after birth, it is common to express the pancreatic glucagon content as ng of glucagon/mg of pancreas (17,19,20) (Fig. 2). In this case, pancreatic glucagon contents were significantly decreased in all strains after weaning as compared to before weaning ($p<10^{-4}$ for C57BL/6 and NOD*scid*; $p<0.05$ for NOD mice). This decreased pancreatic glucagon content that we observed in all strains has been described in other rodents (21,22), and is due to the marked growth of pancreatic tissue, particularly the exocrine compartment. While there was no significant difference in relative pancreatic stored glucagon amounts between the three mouse strains after weaning, before weaning, a difference between control and NOD strains was observed: 7-day-old C57BL/6 mice had significantly higher pancreatic glucagon contents than age-matched NOD ($p<10^{-4}$) and NOD*scid* ($p<0.05$) mice. At 14 days of age, the difference between C57BL/6 and NOD*scid* mice was still significant ($p<0.01$).

Therefore, before weaning, mice with the NOD genetic background are characterized by low circulating glucagon levels and low glucagon pancreatic contents, compared to C57BL/6 mice. These data might suggest low glucagon synthesis in mice with the NOD genetic background before weaning as compared to control mice.

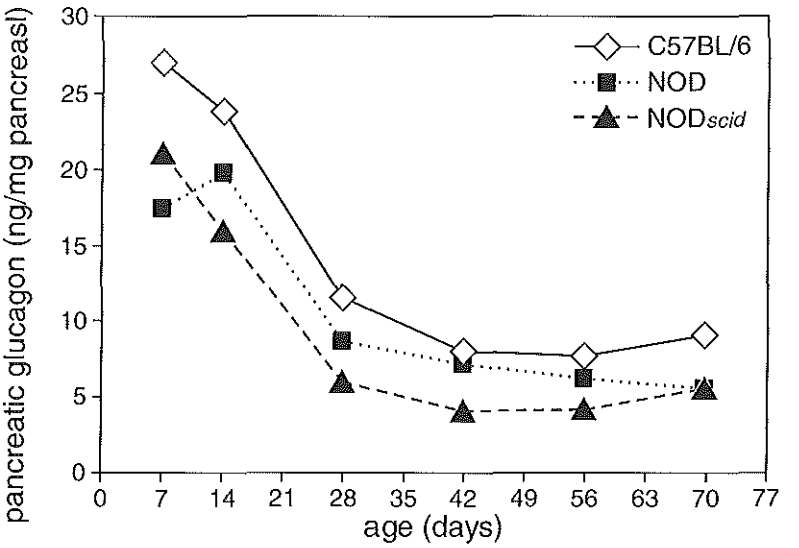

Figure 2: Pancreatic glucagon contents in C57BL/6, NOD and NOD*scid* females as a function of age. Values are expressed as means ± SEM (n=15/age/strain). Individually, strain and age significantly affected pancreatic glucagon contents ($p<10^{-6}$ in both cases) and there was a significant strain x age interaction ($p<0.01$).

NOD mice have a higher α/β cell ratio in their islets than C57BL/6 mice before weaning, especially in very small and small islets

To determine whether the strain-related differences observed in glucagon parameters were reflected in morphological differences at the islet level, we examined islet characteristics combining immunohistochemistry and image analysis to quantify the data.

An example of the immunohistochemical data obtained in pancreata from 7-day-old female C57BL/6 and NOD mice is given in Figure 3. Figure 3a shows a pancreas section from a C57BL/6 mouse stained for insulin and glucagon. At this age, the few glucagon-positive α cells are located at the periphery of large islets surrounding the β cell core, characteristic typical of a mature islet with a low α/β cell ratio. In contrast, sections of age-matched female NOD pancreata (Fig. 3, b and c) show small clusters of glucagon positive α cells, which only occasionally contain insulin-positive β cells. In addition, in NOD mice, many small islets are observed, that seem to have a large proportion of glucagon-positive α cells around the periphery of the β cell core, characteristic of a high α/β cell ratio (Fig. 3d).

We first determined the percentage of islet areas that contained glucagon-producing cells in all islets from C57BL/6, NOD and NOD*scid* mice as a function of age, regardless of their size (Fig. 4a). Considering all ages together, the percentages of glucagon-positive areas in all islets were significantly higher in NOD than in C57BL/6 ($p<0.001$) and NOD*scid* ($p<0.01$) mice (with the latter two being comparable). When comparing NOD and C57BL/6 mice at a given age, the percentages of glucagon-positive areas in islets were significantly

higher in NOD mice only before weaning at 1 and 14 days of age ($p<0.05$) and tended towards significance at 7 and 21 days of age ($0.05<p<0.07$). Thus, before weaning, NOD mice exhibited high α/β cell ratio in their islets, but low glucagon levels in the circulation and pancreas, in contrast to C57BL/6 mice which, at the same age, had the opposite endocrine parameters.

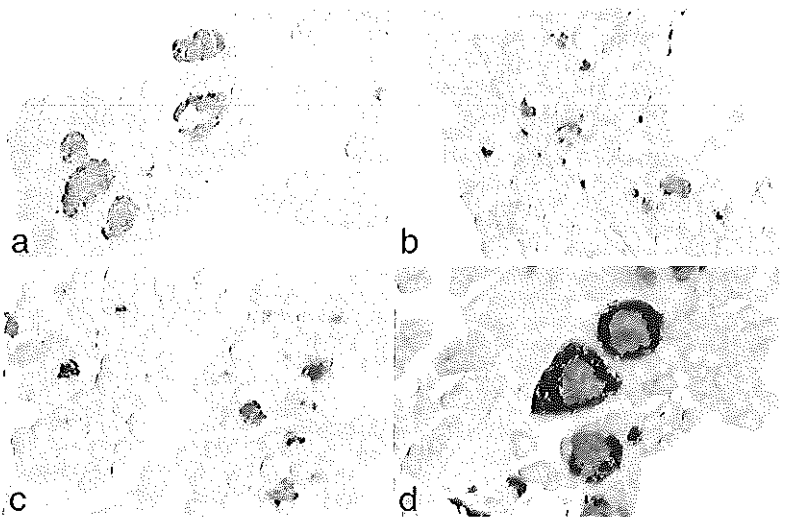

Figure 3: Sections of 7-day-old female C57BL/6 (a) and NOD (b-d) mouse pancreata stained for immunoreactive glucagon (black) and insulin (dark grey) (original magnification a to d: x16 or x25). Small glucagon-positive islets are present throughout the tissue of NOD (b-d) but not C57BL/6 mice (a). Many insulin-containing islets in NOD mice have unusually large proportions of glucagon positive α cells at their periphery (d), as compared to age-matched C57BL/6 (a).

To analyze further the distribution of the glucagon-positive areas in the various strains, we arbitrarily classified islets based on their size: less than 2000 pixels (very small), 2000 to 5000 pixels (small), 5000 to 10000 pixels (medium) and more than 10000 pixels (large). The percentages of glucagon-positive areas were then calculated separately for the different islet size classes. Figure 4b shows that, when considering all ages together, NOD mice had significantly higher percentages of glucagon-positive areas in the very small islets compared to C57BL/6 mice ($p<0.01$), while NOD*scid* mice only showed a trend towards significantly higher values compared to C57BL/6 mice ($p=0.076$). However, NOD and NOD*scid* mice did not differ significantly concerning the percentages of glucagon-positive areas in the very small islets. NOD mice also had higher percentages of glucagon-positive areas compared to C57BL/6 mice in small islets (2000 to 5000 pixels) ($p<0.001$, data not shown), but not in islets of more than 5000 pixels. Thus, the higher percentages of glucagon-positive areas in NOD mice, compared to C57BL/6, are principally accounted for by the very small and small islets.

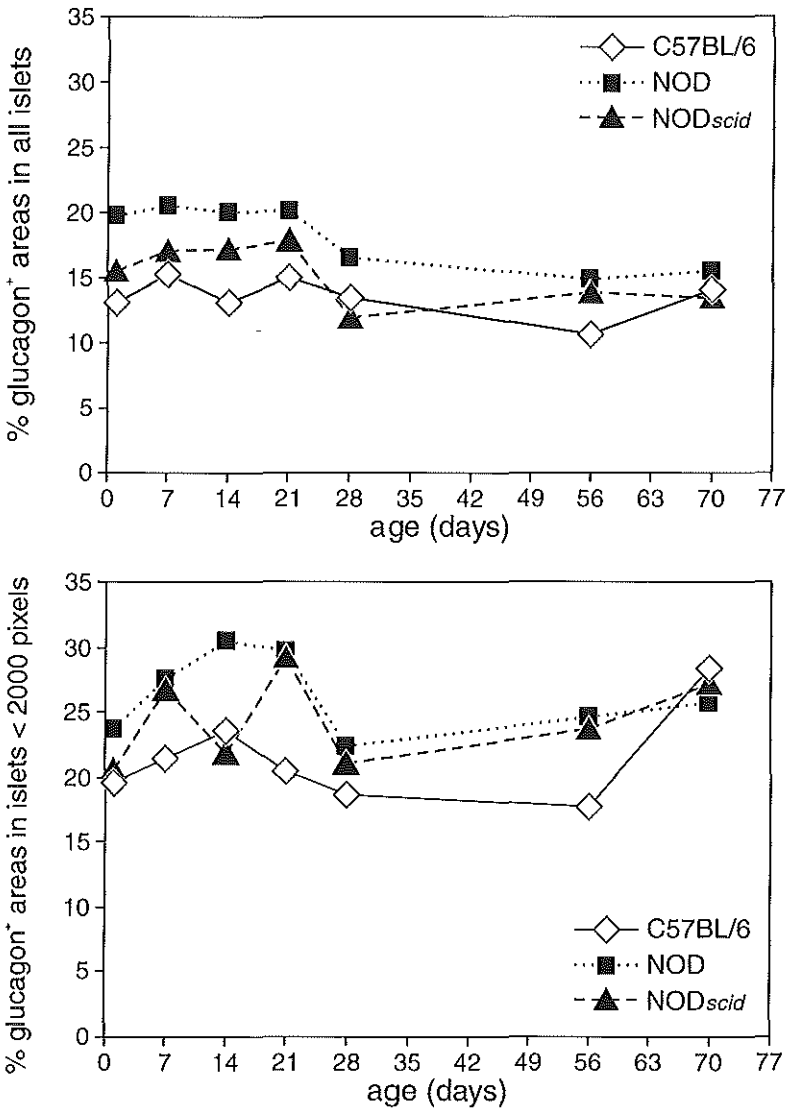

Figure 4: Glucagon-positive areas given as percentages of total islet area in all islets and in very small islets (less than 2000 pixels) in C57BL/6, NOD and NOD*scid* females as a function of age. Values are expressed as means ± SEM (n=6/age/strain). (a) Concerning all islets, both strain ($p<0.001$) and age ($p<0.05$) affected glucagon positivity, but the two variables did not interact significantly. (b) Concerning the very small islets (less than 2000 pixels), ANOVA revealed effects of strain ($p<0.02$) and age ($p<0.05$), but no significant interaction between the two variables.

Both NOD and NOD*scid* mice have more very small islets than C57BL/6 mice during the early postnatal period

So far, we have shown that mice with the NOD genetic background exhibit lower basal glucagon secretion and lower pancreatic glucagon stores than control mice, together with higher α/β cell ratios, particularly in NOD mice. These data suggest the presence of immature islets in NOD mice as schematized in Figure 5, since such islets are characterized by low glucagon synthesis, secretion and content, despite high α/β cell ratios (16-18).

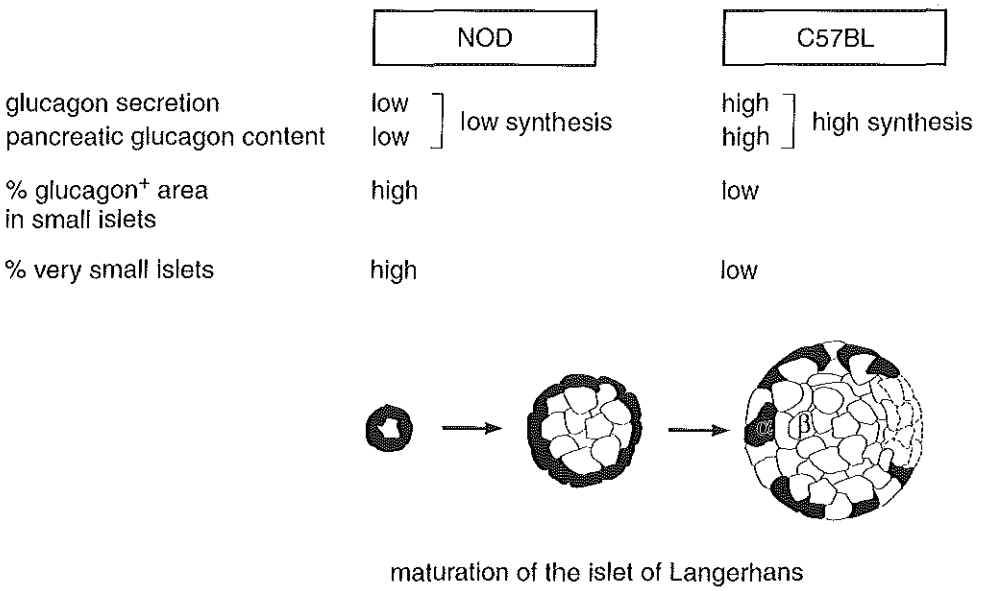

Figure 5: Schema summarizing the different parameters of α cell behavior in NOD and C57BL/6 mice before weaning and their relationship to islet development. Islets of NOD mice exhibit characteristics of immature islets, whereas islets of C57BL/6 mice exhibit characteristics of mature islets.

Finally, we addressed the question whether the numbers of these very small and small immature islets differ between NOD, NOD*scid* and C57BL/6 mice. Indeed, the striking finding was that both NOD and NOD*scid* mice had, on a percentage basis, twice as many very small islets (less than 2000 pixels) as C57BL/6 mice at birth ($p<0.001$) (Fig. 6). A significant difference between strains was still observed at 7 days ($p<0.05$), but had disappeared by 14 days. However, no significant difference was observed between strains for the percentages of small islets.

In summary, before weaning, NOD mice, and to some extent NOD*scid* mice, had more islets with signs of immaturity than C57BL/6 control mice.

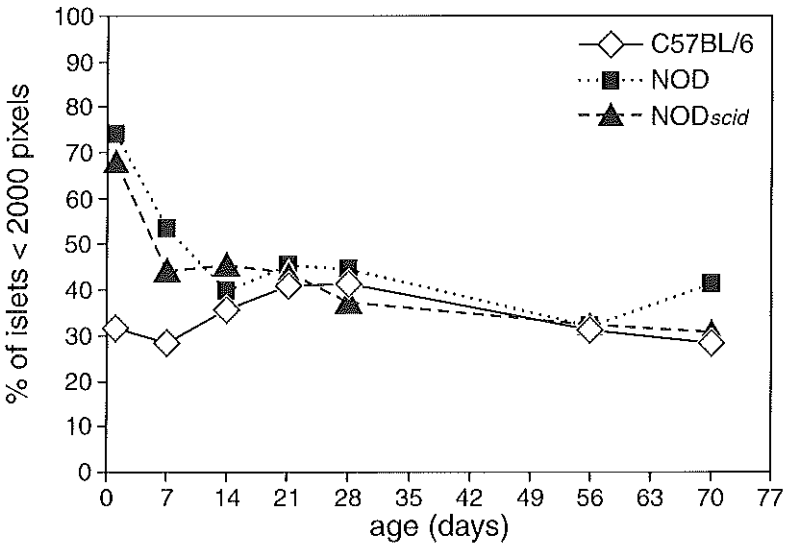

Figure 6: Frequency of very small islets (less than 2000 pixels) in C57BL/6, NOD and NOD*scid* females as a function of age. Values are expressed as means ± SEM (n=6/age/strain). Both strain and age significantly affected the percentages of very small islets ($p<0.01$), but the two variables did not interact significantly.

Discussion

The data presented above illustrate several differences in α cell behavior between mice with the NOD genetic background and control C57BL/6 mice, particularly before weaning. The most conspicuous difference was the presence in 1- and 7-day-old NOD and NOD*scid* mice of high numbers of very small islets with relatively enhanced α/β cell ratios, especially in NOD mice. Moreover, the presence of these small-sized islets is associated, during the early postnatal period, with low blood glucagonemia and low pancreatic glucagon stores. Taken together, these features are characteristic of immature islets and suggestive of perturbed islet development in mice with the NOD genetic background.

Normally, the neogenesis of islets from duct epithelial cells occurs during embryonic development and very early postnatal life (18,23). Normal rodent islet development and/or regeneration is characterized by increased numbers of small islets and frequent connections between islets and ducts (24-26). In the fetal rat, α cells are the most numerous endocrine cell type, accounting for over 2% of the total pancreatic mass at 16 days of gestation (16). The characteristic pattern of central β cells and peripheral non-β cells is not established until day 20 of gestation. By birth, the differential growth rates of the various endocrine cells result in a predominance of β cells, leading to a decreased α/β ratio, while the glucagon content per α cell increases sharply (17).

In normal rodents, glucagonemia and pancreatic glucagon contents are known to be high during the neonatal period and to decline sharply thereafter (14-18). Hyperglucagonemia, immediately after birth, is considered to be an adaptative mechanism to counterbalance the normally occurring neonatal hypoglycemia (14,15) and was observed here before weaning in C57BL/6 mice, but also in all normal mouse strains we investigated (unpublished results). It is worth noting here that basal glucagonemia before weaning was significantly lower in mice with the NOD genetic background than in C57BL/6 mice, concomitant with low glucagon contents and high numbers of very small islets with a high α/β ratio, as assessed immunohistochemically. According to what is known about islet differentiation, these characteristics are suggestive of the presence of more immature islets in NOD (and also to some extent NOD*scid*) mice than in C57BL/6 (Figure 5).

Later, i.e., after weaning, NOD and NOD*scid* mice experienced relative hyperglucagonemia, thereby confirming the results obtained by others (13). This hyperglucagonemia might be due to an endocrine imbalance at the islet level or be attributable to the increasing numbers of leukocytes around the islets. For example, cytokines, such as interleukin-1, are known to stimulate glucagon secretion in vivo and in vitro (27-29). Recently, we observed that interleukin-1 was able to stimulate in vivo glucagon secretion in prediabetic NOD females (unpublished results). Moreover, since glucagon is known to stimulate insulin secretion (10-12), it cannot be excluded that the relative hyperglucagonemia postweaning might be partly responsible for the basal hyperinsulinemia that we previously observed, particularly in NOD females, from 4 weeks of age onwards (5). Such a sequence of events has already been described in C57BL/Ks mice, a spontaneous model of type 2 diabetes (12).

In light of the concepts described above, our data suggest that newborn NOD mice, and also to some extent NOD*scid* mice, harbor more immature islets with features characteristic of neogenesis, i.e., very small size associated with large glucagon-positive areas and diminished capacities to synthesize and secrete glucagon. Other investigators have also described postnatal developmental islet abnormalities in the NOD mouse. For example, very high percentages of adult β stem cells ($SOM^+/PDX1^+$) were found in 4-week-old NOD mice compared to ICR mice (40% versus 10%, respectively) (30). The numbers of these stem cells increased thereafter in parallel with the mononuclear infiltration. It should be underlined that, in NOD and NOD*scid* mice, the early islet infiltration occurs at the islet periphery where α cells are located (4,7). This situation is intriguing, since the normal blood flow within the islet follows a β to α direction (31). Therefore, infiltrating leukocytes would not have to pass the α cells at the islet periphery to reach the β cells, which are considered to be the main target of the autoimmune reaction. While there is some evidence that α cells themselves might also be a target in type 1 diabetes (32-35), they appear to be spared from autoimmune destruction. This selective protection might be due to the expression of the Fas-ligand on the α cells of normal mice and prediabetic NOD mice (36).

In conclusion, the disturbances that we observed in NOD α cell neogenesis might reflect subtle abnormalities in islet development, which may cause or stem from β cell dysfunction, resulting in increased β cell sensitivity or even targeting of the autoimmune reaction. Intriguingly, in this regard, delayed development of the salivary glands, another target of the autoimmune reaction in mice with the NOD genetic background, has also been described recently (Humphreys-Beher et al., personal communication). These observations point to a previously unrecognized role for organ development in the triggering of an autoimmune disease. In addition to the essential defects in immune function that determine the susceptibility to the development of autoimmunity, such developmental target-organ abnormalities may be involved in the tissue specificity of autoimmune diseases, like autoimmune diabetes.

Materials and methods

Animals

NOD, originally provided by Clea Japan Inc. (Tokyo, Japan), NOD*scid* and C57BL/6 females were bred at the animal facilities of Hôpital Necker, Paris, France, under specific pathogen-free conditions, according to the norms stipulated by the European Community. They were antibody-free for the 13 viruses tested, including diabetogenic viruses (37). Mice were fed standard pellets and water ad libitum and maintained at 22°C on a 12 h light - dark cycle. Nondiabetic NOD females (with basal nonfasting glycemia < 11 mmol/l, as assessed by Glukotest, Boehringer-Mannheim, Mannheim, Germany) were used. In our NOD colony, during the time of this investigation, overt diabetes appeared in females from the 12^{th} week of age onwards and 80% of them had become diabetic by 6 months of age.

Measurement of blood and pancreatic glucagon

In all cases, unanesthetized animals were bled in less than 2 min by retroorbital puncture. As previously shown, this technique avoids stress-induced metabolic changes, at least during the sampling time (38). In each series of experiments, different groups of animals of various ages (7, 14, 28, 42, 56 and 70 days old) were bled at different times to avoid the hyperglycemic effect of repeated orbital puncture (38). Blood samples were supplemented with recombinant aprotinin, 30,000 kIU/ml (Bayer Inc., Kankakee, IL,), kept on ice, centrifuged at 13,000 x g for 2 min at 4°C, and stored at -20°C. Mice were then killed by cervical dislocation. Pancreata were rapidly removed, weighed and homogenized in 15 ml of cold acid ethanol extraction medium (1.5% (v/v) 1 N HCl in 75% ethanol). After addition of another 10 ml of extraction medium, the homogenates were centrifuged (800 x g for 15 min, 4°C), and the supernatants were left standing overnight at 4°C. The pH of the supernatants was

adjusted to 8.5 with ammonium hydroxide and, after centrifugation (800 x g for 15 min, 4°C), 5 ml of each supernatant were stored at - 20°C until assayed. Glucagon concentrations were determined using a standard radioimmunoassay kit (rabbit anti-human glucagon, Biodata, Pharmacia, Saint-Quentin-en-Yvelines, France). Pancreatic glucagon contents are expressed as ng/mg of pancreas, as commonly used (17,19,20).

Immunohistochemistry

Six pancreata for each strain were removed at 1, 7, 14, 21, 28, 56 and 70 days of age. After excision, they were immediately embedded in OCT (Tissue-Tek, Miles, Elkart, IN), frozen in dry ice-cooled isopentane and stored at -80°C until sectioning. Cryostat sections, 6-µm thick, were cut, dried overnight and frozen at -20°C until being subjected to labeling. Tissues used for double-labeling experiments were fixed in Bouin's solution (Sigma, Saint-Quentin-Fallavier, France) and paraffin-embedded; 4µm-thick sections were cut.

Sections were allowed to thaw at room temperature for 30 min. After being fixed in acetone for 10 min, slides were washed in phosphate-buffered saline (PBS) - 0.05% Tween 20 (Tw). Sections were incubated with prediluted rabbit anti-human glucagon (Dako, Trappes, France) for 30 min. After washing in PBS-Tw, sections were incubated with the secondary antibody, biotinylated swine anti-rabbit Ig (Dako), diluted 1/100, for 30 min. After rinsing in PBS-Tw, Avidin-Biotin Complex (Vectastain kit, Vector, Burlingame, CA) was added for 20 min. After further washing, the bound complex was developed with 3-amino-9-ethyl-carbazole (Sigma) in 50 mM sodium acetate. After 2.5 min, the reaction was stopped with distilled water. After rinsing in PBS-Tw, sections were counterstained with Mayer's hemalum solution (Merck, Paris, France) and mounted in Faramount aqueous mounting medium (Dako). Negative controls were performed with reagents supplied by Dako.

Double labeling was performed on 4-µm sections cut from paraffin-embedded tissues. After dewaxing and rehydration, the tissues were washed in Tris-saline buffer (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.05% Tw). Sections were incubated with optimally diluted guinea pig anti-insulin serum (Linco Research Inc., St. Charles, MO) and rabbit anti-glucagon serum (Chemicon International Inc, Temecula, CA) overnight at 4°C. Slides were thoroughly washed in Tris-saline buffer and incubated first with alkaline phosphatase-conjugated swine anti-rabbit IgG (Dako) for 30 min at room temperature, rinsed and then incubated with rabbit anti-guinea pig IgG coupled to horseradish peroxidase (Dako) for a further 30 min. Sections were rinsed first in Tris- saline buffer (pH 7.5), then briefly in alkaline Tris-saline buffer (pH 9.5 containing 50 mM $MgCl_2$). Immunoreactive glucagon was revealed using a substrate consisting of 0.34 mg/ml of nitroblue tetrazolium chloride (Boehringer Mannheim) and 0.18 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate (Research Organics Inc., Cleveland, OH). Slides were protected from light and periodically checked for staining intensity. The reaction was stopped by returning sections to neutral pH in Tris-saline buffer containing 10 mM EDTA. Slides were washed thoroughly for at least 30 min, then reacted with

the peroxidase chromogen diaminobenzidine (Sigma) in PBS with 0.02% H_2O_2 for 1 to 2 min. Slides were finally rinsed in water, counterstained with methyl green (Fluka, Buchs, Switzerland), progressively dehydrated through alcohol baths, cleared, and mounted.

Quantification of immunohistochemistry

All islets from each glucagon-labeled section were considered. The area of each islet was measured using a VIDAS-RT image analysis system (Kontron Elektronik GmbH/Carl Zeiss, Weesp, The Netherlands). The counterstaining enabled easy identification of the islets which were encircled by hand and then the computer program automatically calculated the area of each islet in pixels, and determined the glucagon-positive area and the percentage of glucagon-positive area of each islet. Measurements were made at a magnification of 100x, at which the size of one pixel is 1.13×10^{-6} mm^2.

For the analysis of some data, islets were arbitrarily classified into various groups according to their size: very small (area less than 2000 pixels), small (2000 to 5000 pixels), medium (5000 to 10000 pixels) and large (more than 10000 pixels). The percentages of glucagon-positive areas were then determined for each class of islets.

The total number of islets was counted for a strain at a given age (one section per mouse and per age, n=6 mice for each age group of a given strain). The total numbers of islets were: on day 1 (78-95), day 7 (119-154), day 14 (119-181), day 21 (138-175), day 28 (85-110), day 56 (68-102) and day 70 (100-203).

Statistical analyses

All data are expressed as means ± SEM. Possible effects of strain and age were analyzed using ANOVA for each variable investigated. Post hoc analysis, using the Newman-Keuls test, was performed when effects and interactions were significant ($p<0.05$), as assessed by ANOVA. When the interaction was not significant, planned comparisons were made.

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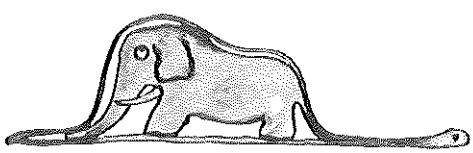
Chapter 3.6

Neonatal pancreatic infiltration by dendritic cells and macrophages in mice with the nonobese diabetic (NOD) or non-autoimmune genetic background



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Summary

In a few species, macrophages (Mϕ) and dendritic cells (DC) have been observed in the pancreas during early stages of normal development. This finding is possibly relevant for the development of type 1 diabetes, in which these cells are thought to be the first infiltrating cells around weaning, then leading to insulitis development. However, the presence of these antigen-presenting cells (APC) during postnatal pancreas growth in control and diabetes-prone mouse strains has never been investigated.

Here, we compare during the first month of life the numbers of APC ($CD11c^+$ DC, ER-$MP23^+$ and $BM8^+$ Mϕ) in three control strains and in NOD and NOD*scid* mice. The latter lack functional lymphocytes and do not progress to diabetes in contrast to NOD mice. In mice with the NOD genetic background, inflammatory APC infiltration is usually thought to start around weaning (3 weeks of age). We show that low numbers of $CD11c^+$ DC are present in control postnatal pancreata, while enhanced numbers are observed in NOD and NOD*scid* mice from birth onwards. NOD mice also exhibit higher numbers of ER-$MP23^+$ Mϕ at birth compared to control strains. Finally, high numbers of $BM8^+$ Mϕ are observed at birth in all strains investigated These cells disappear more or less progressively during the first month of life. APC-locations are similar in all strains, i.e., in peri-vascular, peri-ductular, peri-islet areas and in septa.

In conclusion, the inflammatory lesions observed in NOD mice after weaning resemble phenomena observed transiently during normal postnatal pancreas development.

Introduction

Type 1 (insulin-dependent) diabetes mellitus (type 1 diabetes) is the result of an autoimmune destruction of the β cells in the pancreatic islets of Langerhans. In the spontaneous models of the disease, the NOD mouse and the bio-breeding (BB) rat, the infiltration of macrophages (Mϕ), dendritic cells (DC) and lymphocytes around the islets of Langerhans is considered to be the most precocious lesion (1-4). In this so-called peri-insulitis, Mϕ and DC likely function as antigen-presenting cells. In NOD females, $CD11c^+$ DC have been shown to accumulate from 3-5 weeks of age onwards around and within the islets of Langerhans (5,6). At the same time, ER-$MP23^+$ Mϕ, that are normally present in the NOD pancreas connective tissue, migrate to the periphery of the islets with some cells penetrating into them (1). The early APC accumulation plays an important role in the development of insulitis, since prevention of early monocyte influx stops the autoimmune process and protects from diabetes development (7). It is only after the early DC and ER-$MP23^+$Mϕ accumulation, i.e., from 7-10 weeks of age onwards, that large numbers of T and B lymphocytes and $BM8^+$ scavenger Mϕ are recruited to the islets in NOD females (1).

DC and Mϕ are also often found around the ducts, especially in early stages of the disease (1,8-10). The relationship between peri-ductular infiltration and insulitis is still largely obscure. However, it should be noted that the islets of Langerhans originate from the ductal epithelial cells (11). In this regard, an association between phenomena taking place at the duct and/or the islet level (such as neogenesis and regeneration) and infiltration involving Mϕ and/or lymphocytes has been observed in several situations, not necessarily associated with autoimmune autoimmune diabetes. For example, in an animal model of non-autoimmune type 2 diabetes, the OLETF rat, a moderate leukocyte infiltration exists in and around the islets of Langerhans in young rats (12). This infiltration is followed by fibrosis of the islets and hyperplasia of the pancreatic ducts after 12 weeks. Inflammatory infiltrates within the pancreas and insulitis have also been observed in several situations involving (auto-)immune responses (13,14), and in various mice transgenic for cytokines or growth factors, some of the latter associated with strong pancreatic regeneration (15,16). Moreover, Mϕ play a major role in the processes of pancreatic degeneration and regeneration which take place after duct ligation-induced pancreatitis (17,18). Finally, an association between pancreas growth and the presence of leukocytes also exists in humans. Early studies have noted a lymphocytic infiltration during normal human fetal pancreas development concomitant to degeneration of the islets (19). More recent data confirm the presence of leukocyte infiltrates in the exocrine pancreas, including a few DC and lymphocytes in the islet vicinity, as a normal feature of the fetal and neonatal human pancreata (20-23). Taken together, these observations in various species suggest that leukocytes may play a role during normal pancreas development or regeneration.

In normal mouse strains, however, the presence of APC in the developing pancreas has never been investigated. Pancreas development, including islet differentiation from ducts (neogenesis), takes place in control mouse strains at least until weaning (3 weeks of age) (24). During the postnatal period, apoptosis of β cells and other cell types is observed (25). Therefore, it can not be excluded that leukocytes are normal but transient constituents of the developing pancreas. We therefore analyzed from 1 day to 4 weeks of age, the numbers and localization of $CD11c^+$ DC and ER-$MP23^+$ and $BM8^+$ Mϕ in the pancreata of various control strains (C57BL/6, DBA/2 and BALB/c). We also approached the question whether early postnatal APC infiltrates are altered in mice with the NOD genetic background compared to control strains and we therefore studied their presence in NOD and NOD*scid* strains. The latter lacks functional lymphocytes and does not progress to full insulitis and diabetes (26), but shares with the NOD mouse the early APC infiltration (5). Our data show that a transient APC infiltration is a normal feature of the postnatal developing mouse pancreas, regularly associated with islet neogenesis. Several differences have been observed between normal mouse strains and diabetes-prone NOD mice, in terms of kinetics and APC numbers but not localization.

Results

Low densities of $CD11c^+$ DC are present in control pancreata, whereas enhanced numbers are present in NOD and NOD*scid* mice from birth onwards

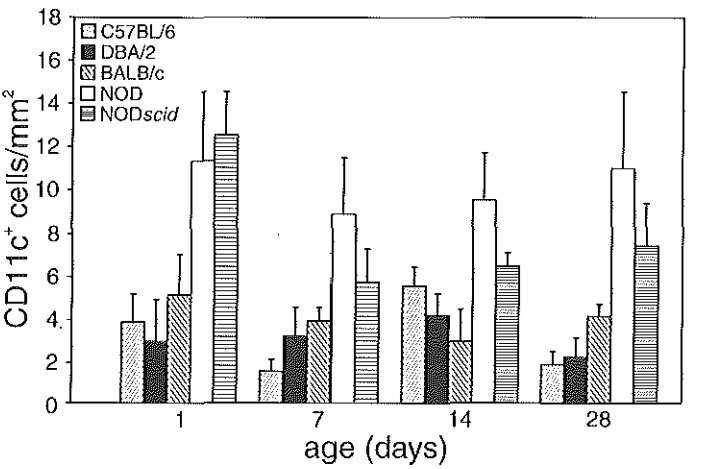

Figure 1: Numbers of $CD11c^+$ DC in pancreata of various mouse strains as a function of age. The numbers of cells were counted and expressed per mm^2 of total pancreatic surface area in each strain, as assessed by image analysis. A group of 5 mice (one section per mouse was analysed per age-strain (mean values ± SEM). $CD11c^+$ DC are present in higher numbers in NOD and NOD*scid* mouse pancreata than in controls.

Figure 1 shows that $CD11c^+$ DC were found in the pancreata of all strains investigated during the postnatal period, but in very low numbers. The numbers of $CD11c^+$ DC were stable in a given strain during the first four weeks of age. There was, however, a difference between the strains ($p<10^{-6}$, as assessed by ANOVA). Higher densities of $CD11c^+$ DC were observed consistently from birth onwards in strains with the NOD genetic background. This effect was significant, at 1 day of age, for NOD*scid* compared to C57BL/6 and DBA/2 mice (p=0.03 and p=0.01, respectively, by *post-hoc* analyses) and tended towards significance for NOD versus DBA/2 and NOD*scid* versus BALB/c mice (p=0.06 and p=0.08, respectively). At 4 weeks of age, $CD11c^+$ DC values were significantly higher in NOD compared to C57BL/6 and DBA/2 mice (p=0.025 and p=0.035, respectively). These differences represent the start of the classical insulitis in NOD mice (5,6).

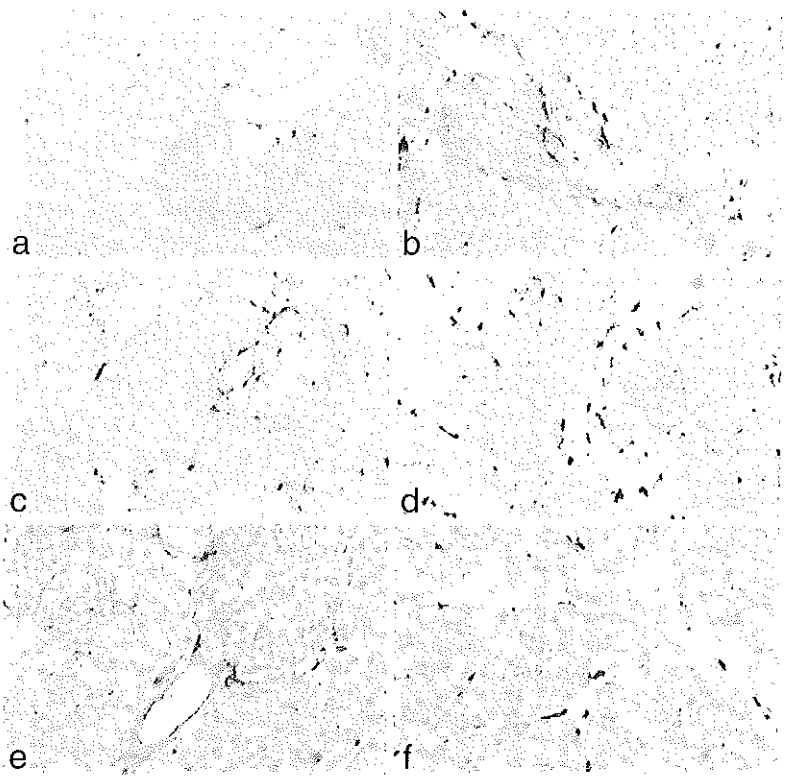

Figure 2: Immunohistochemistry for $CD11c^+$ DC, ER-$MP23^+$ and $BM8^+$ Mϕ in control strain pancreata. (a) $CD11c^+$ DC in 4-week-old DBA/2 mouse (original magnification x16); (b) ER-$MP23^+$ Mϕ in a 2-week-old C57BL/6 (original magnification x16); (c and d) ER-$MP23^+$ Mϕ in 4-week-old C57BL/6 and BALB/c mice (original magnification x16); (e and f) $BM8^+$ Mϕ in 4-week-old C57BL/6 (original magnification x25). Regardless of the cell type, locations in peri-vascular, peri-ductular and peri-islet areas and septas are observed in control pancreata.

Concerning their localization, in all mouse strains the few CD11c$^+$ DC appeared to be scattered throughout the pancreas at the youngest ages investigated, when the immaturity of the pancreas was still obvious. Then, they were progressively found around vessels, ducts and islets, even in normal strains (Fig. 2a). At 4 weeks of age, however, CD11c$^+$ DC could be observed inside the islets in NOD mice, whereas they stayed around the islets in control mice.

High densities of ER-MP23$^+$ Mϕ are present in control pancreata, whereas enhanced numbers are present in NOD neonates

As shown in Figure 3, relatively high numbers of ER-MP23$^+$ Mϕ were observed in all strains, at least during the first 3 weeks, followed by a drop at 4 weeks of age. This age effect turned out to be highly significant ($p<10^{-6}$, ANOVA). Numbers obtained in 4-week-old mice were significantly lower compared to those obtained at 1 day of age for C57BL/6, DBA/2, BALB/c and NOD mice, at 1 week of age for C57BL/6, NOD and NOD*scid* mice, and at 2 weeks of age for NOD and NOD*scid* (p values from 0.05 to 0.0001, *post-hoc* analyses). Moreover, there were also differences between the strains (p=0.02, ANOVA). At 1 day of age, significantly higher numbers of ER-MP23$^+$ Mϕ were observed in NOD mice compared to all other strains, including NOD*scid* (p values from 0.02 to 0.0002, *post-hoc* analyses).

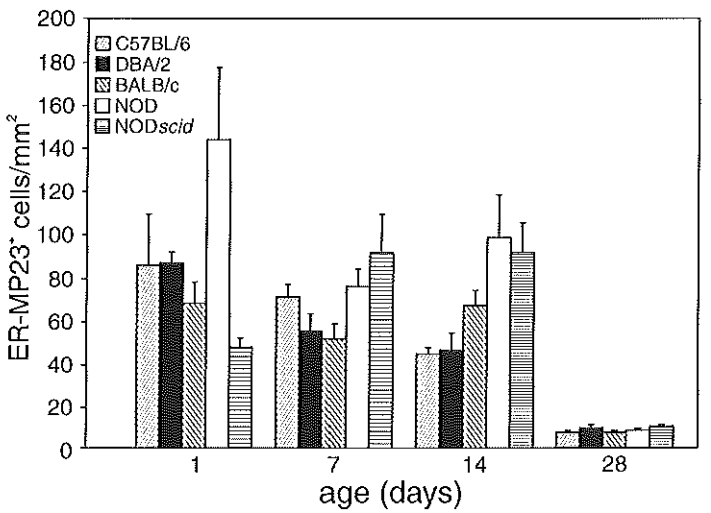

Figure 3: Numbers of ER-MP23$^+$ Mϕ in pancreata of various mouse strains as a function of age. Results are expressed as in Figure 1. High numbers of ER-MP23$^+$ Mϕ are present in control pancreata during the postnatal period, but enhanced numbers are present in 1-day-old NOD neonates.

With regard to the localization, ER-MP23$^+$ Mϕ appeared rather dispersed within the pancreas at 1 day and 1 week of age. Typically, these Mϕ were embedded in the connective tissue separating the epithelial compartments. Beyond 1 week of age, ER-MP23$^+$ Mϕ were observed, particularly, in peri-vascular, peri-ductular and peri-islet areas as well as in inter-lobular areas in all strains (Fig. 2b,c,d). In 4-week-old NOD mice but not in other strains, some ER-MP23$^+$ Mϕ could also be found inside the islets, as was the case for CD11c$^+$ DC.

A decrease in BM8$^+$ Mϕ density occurs in all strains after birth

Mϕ, that is observed in control strains, is delayed in NOD and NODsicd mice. The BM8$^+$ Mϕ represent a third leukocyte type present in the pancreas in early postnatal phases. As was observed for ER-MP23$^+$ Mϕ, relatively high numbers of BM8$^+$ Mϕ were observed shortly after birth (Fig. 4). The numbers of BM8$^+$ Mϕ decreased significantly with increasing age ($p< 10^{-6}$, ANOVA). Interestingly, this decrease appeared earlier in control strains than in strains with the NOD genetic background. The decrease was already significant between 1 day of age and 1 week of age for C57BL/6 and DBA/2 ($p=0.01$ and $p=0.0001$, respectively, post-hoc analyses), or 1 week and 2 weeks of age for BALB/c ($p=0.01$). In NOD and NOD*scid* mice, a significant decrease in the number of BM8$^+$ Mϕ was also observed, but this occurred later in time, between 2 and 4 weeks of age ($p=0.03$ and $p=0.0001$, respectively).

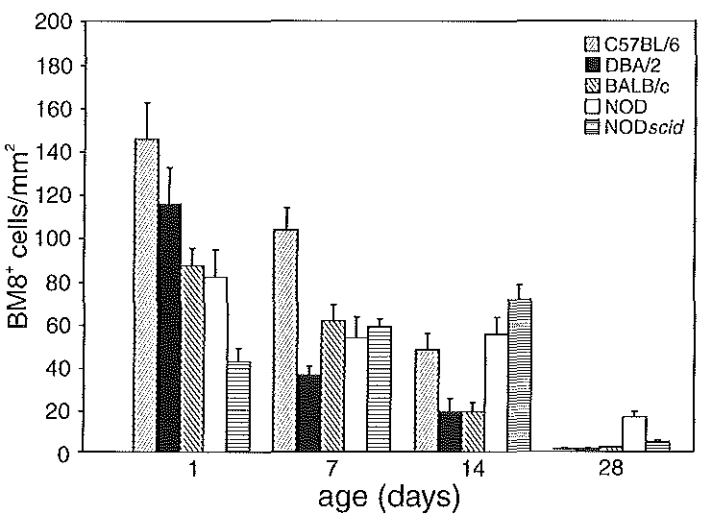

Figure 4: Numbers of BM8$^+$ Mϕ in pancreata of various mouse strains as a function of age. Results are expressed as in Figure 1. Note the highest numbers of BM8$^+$ Mϕ at birth in pancreata of all strains except NOD*scid*.

Moreover, at a given age, there were many significant differences between the various strains concerning density of $BM8^+$ Mϕ. The most pertinent differences were the following: C57BL/6 showed the highest levels of $BM8^+$ Mϕ observed at 1 day and 1 week of age compared to all other strains (p values between 0.005 and 0.0001, post-hoc analyses) and, NOD*scid* contained, at 1 day of age, the lowest levels compared to all other strains, including NOD (p values from 0.03 to 0.0001). At 2 weeks of age, NOD and NOD*scid* mice exhibited significantly higher numbers of $BM8^+$ Mϕ than two of the control strains (DBA/2 and BALB/c, p values between 0.04 and 0.001), but this effect was found to be no longer significant at 4 weeks of age, the time of maximal decrease of $BM8^+$ Mϕ numbers in all strains.

Considering their localization, $BM8^+$ Mϕ appeared to be either scattered throughout the pancreas or already grouped together in peri-islet or peri-acinar areas at birth. Thereafter, they were commonly observed in peri-vascular, peri-ductular and per-islet areas and septa in all strains (Fig. 2e,f). Remarkably, $BM8^+$ Mϕ were also sometimes localized at the places of islet neogenesis, i.e. the differentiation of islets from ducts. An almost continuous line of $BM8^+$ Mϕ surrounded the ducts and the islets and appeared to accumulate at the interface between both structures (Fig. 2e). Such pictures were regularly observed in all strains until 4 weeks of age.

APC are present in the same locations in mice with the NOD genetic background and control mice

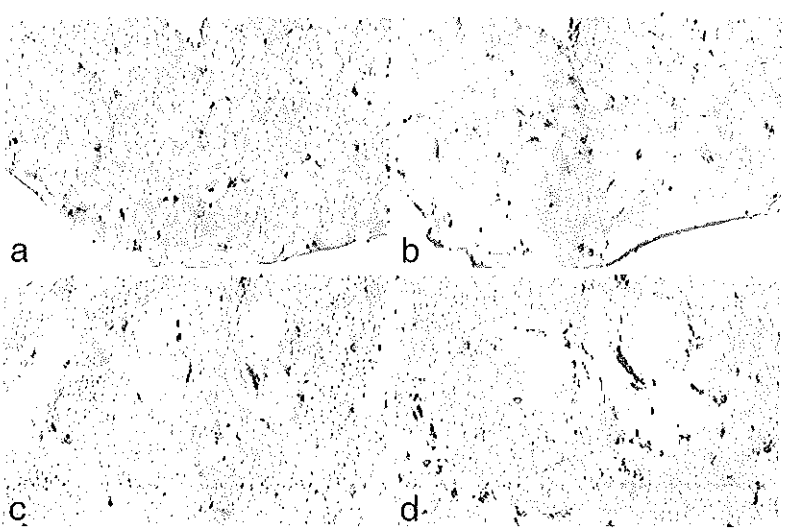

Figure 5: Serial staining for ER-$MP23^+$ Mϕ (a,c) and $BM8^+$ Mϕ (b,d) in the pancreas of a 1-day-old NOD mouse. The locations of ER-$MP23^+$ and $BM8^+$ Mϕ are not different in postnatal NOD and control pancreata (Fig. 2).

Serial stainings were done to assess the localization of both types of Mϕ in the NOD after birth. Figure 5 shows two examples of staining for ER-MP23$^+$ and BM8$^+$ Mϕ on serial pancreatic sections from 1-day-old NOD mice. The first example (Fig. 5a and b) illustrates that both types of cells are scattered throughout the exocrine pancreas, in particular in peri-acinar localization. Most of the time (if not always), ER-MP23$^+$ and BM8$^+$ Mϕ do not colocalize. The second sample (Fig. 5c and d) shows both cell types in peri-vascular, peri-ductular and peri-islet areas. ER-MP23$^+$ and BM8$^+$ Mϕ can be observed close to the same structures, in particular the peri-vascular-ductular pole of the islet. However, when scattered in the pancreas, both stainings do not colocalize. Moreover, when Figures 2 (showing data obtained in control mice) and 5 (concerning NOD mice) are compared, it appears that the locations of ER-MP23$^+$ and BM8$^+$ Mϕ in the postnatal period are not different between control and NOD mice.

Discussion

This study shows the presence of a transient APC infiltration as a normal feature of the developing pancreas. In postnatal pancreata, low numbers of CD11c$^+$ DC and relatively high numbers of BM8$^+$ and ER-MP23$^+$ Mϕ are present. However, a strong decrease in both cell types takes place after weaning in these strains. The localization of these Mϕ in normal strains is strikingly similar to that classically described in NOD mice during early insulitis after weaning.

Among the DC and Mϕ subsets present in normal strains, the most remarkable data concern the presence and localization of BM8$^+$ Mϕ. These cells are present in high numbers at birth and progressively decline during the first month of life. At 4 weeks of age, i.e., after weaning, virtually no BM8$^+$ Mϕ are detectable in all control strains, confirming previous data (1). ER-MP23$^+$ Mϕ are also present at relatively high levels in normal strains from birth onwards, but their numbers are quite similar among all these strains and stable during the first two weeks of life. A decrease is observed at 4 weeks of age, but low numbers of ER-MP23$^+$ Mϕ persist, which is consistent with their presence in normal adult mouse pancreas (1). CD11c$^+$ DC are observed in control strains at low and stable numbers during the first month of life. It is worth noting here that the decline of high numbers of BM8$^+$ and ER-MP23$^+$ Mϕ correlates in time with the completion of postnatal pancreas development (11,24,25).

In terms of cell densities, some differences are observed between control strains and strains with the NOD genetic background. In contrast to control strains, NOD strains tend to have higher numbers of APC as early as birth. This is the case for ER-MP23$^+$ Mϕ in NOD mice and CD11c$^+$ DC in NOD and NOD*scid* mice. The presence of increased numbers of some types of APC as early as birth in mice with the NOD genetic background should be underlined, since the classical peri-insulitis is assumed to start at about 3 weeks of age (1).

In this regard, an ongoing autoimmune reaction in the NOD fetus, which could be responsible for increased local APC densities, has already been postulated on the basis of an *in utero* T cell activation (27). Another intriguing strain difference in terms of cell densities at birth is observed between NOD and NOD*scid* mice: the latter showed significantly lower numbers of ER-MP23$^+$ and BM8$^+$ Mϕ compared to NOD mice at 1 day of age. The reason for this lower APC number in the pancreas is unclear. Possibly, APC number and/or function could be decreased in NOD*scid* mice due to relative immaturity of their APC as a result of absence of functional lymphocytes. This is illustrated by the finding that APC isolated from NOD*scid* islets are much less effective in stimulating diabetogenic T cell clones than those isolated from NOD islets (28). In addition, transfer of T cell clones *in vivo* improves the antigen-presenting function of NOD*scid* APC (28).

Another strain difference between control mice and mice with the NOD genetic background concerns the kinetics of cell disappearance. In particular, NOD and NOD*scid* mice show stable BM8$^+$ Mϕ numbers during the two first weeks of age followed by a decrease at 4 weeks of age. The decline in BM8$^+$ Mϕ in these strains is not as progressive as that observed in control strains and appears delayed. The reason of the abnormal BM8$^+$ Mϕ kinetics remains unknown. Several genetic and functional defects have been described at the APC level in NOD mice. These defects include defective FcγRII gene expression, deficient differentiation and function, such as antigen presentation, decreased cytokine secretion and enhanced arachidonic acid metabolism (29-33). BM8$^+$ Mϕ are known to reappear later during the progression of insulitis in NOD mice and are thought to play a critical role in the final destruction of the islets (1,34). It could be suggested that the delayed BM8$^+$ Mϕ decrease reflects an early influx of inflammatory cells.

The second intriguing result of this work is that APC in control postnatal pancreas are present in similar locations (i.e., peri-vascular, peri-ductular and peri-insular) as are the first infiltrating APC in spontaneous animal models of type 1 diabetes (1,35,36). The peri-ductular localization of APC and lymphocytes has been repeatedly noted in these models (1,8-10), but the reason for this infiltration is still poorly understood. Our present data on the presence of leukocytes in neonatal control animals might shed light on this phenomenon. Tissue remodeling phenomena (islet neogenesis, β cell replication and apoptosis of various cell types) exist in the pancreas of normal rodents, and particularly in mice until weaning (24,25). At present, nothing is known about the mechanisms occurring during islet neogenesis, which lead to the detachment of the islet from the duct and the rupture of the basal membrane surrounding both structures. The processes through which pancreatic interlobular septa develop are unknown. APC might be normally involved in these developmental processes, since they have a well-recognized role in tissue remodeling (synthesis and degradation of extracellular matrix) and apoptosis induction (37-39). Moreover, DC and Mϕ are also able to produce many of the cytokines and growth factors known to be implicated in normal pancreas development (11,40). It is worth noting that we show here that APC are especially present in remodeling tissue locations in control mice as well as in mice with the NOD genetic back-

ground during the postnatal period. These non-lymphoid cells, functioning as APC, might also be involved in the induction of peripheral immune tolerance to self (neo?)antigens expressed during pancreas development (41).

In conclusion, myeloid cells can be found in the postnatally developing pancreas of control mouse strains as well as strains with the NOD genetic background. NOD mice, however, exhibit higher numbers of some types of these cells as early as 1 day of age, although the classical peri-insulitis/insulitis is usually thought to start after 3 weeks of age. The location of leukocytes in neonatal pancreas is similar in all strains investigated, in particular in peri-ductular and peri-islet areas of islets. It is worth noting that lesions observed in NOD mice after weaning resembled, to some extent, phenomena normally observed in control mice during the postnatal period.

Materials and methods

Animals

NOD, NOD*scid*, C57BL/6, DBA/2 and BALB/c female mice were bred under specific pathogen-free conditions at the facilities of the Hôpital Necker, Paris, France, as previously described (42). The animal facilities and care followed the norms stipulated by the European Community. The incidence of diabetes in the NOD colony is, by 200 days of age, 80% for females (42). Mice of different strains were sacrificed at 1 day, and 1, 2 and 4 weeks of age for evaluation of the DC and Mϕ infiltration.

Antibodies

N418, a hamster-anti-mouse CD11c antibody, was used to identify DC (43) and ER-MP23, a rat-anti-mouse Mϕ galactose-specific lectin (MMGL) was used to detect a particular subset of Mϕ (43). Both antibodies were used as undiluted hybridoma culture supernatants. BM8, a rat anti-mouse antibody, identifying a 125 kD cell surface protein of scavenger macrophages (43), was obtained from BMA (BMA Biomedicals AG, Augst, Switzerland) and diluted 1/50. BM8 and ER-MP23 binding was detected with horseradish peroxidase (HRP)-conjugated rabbit-anti-rat immunoglobulins (Dako, Glostrup, Denmark) diluted at 1/100. N418 was detected with HRP-conjugated goat-anti-hamster immunoglobulins (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania, USA) diluted at 1/200.

Immunohistochemistry

Mice of different strains were killed by cervical dislocation after rapid retro-orbital bleeding. Their pancreata were removed, embedded in OCT (Tissue-Tek, Miles, Elkart, IN)

compound and frozen in n-hexane on dry-ice chilled alcohol. Tissues were stored at -80°C until immunohistochemistry was performed. Pancreas cryostat sections of 6 mm were fixed for 10 min in acetone. After a wash with phosphate-buffered saline with 0.05% Tween-20 (Merck, Paris, France) (PBS/Tween), slides were incubated with first step monoclonal antibodies for 30 min at room temperature in a moist chamber. Subsequently, slides were washed twice with PBS/Tween and then incubated with second step antibodies in the presence of 2% normal mouse serum for 30 min at room temperature. After an additional wash with PBS/Tween, slides were incubated with_3-amino-9-ethylcarbazole (AEC, Sigma, Saint-Quentin-Fallavier, France) as substrate in 50 mM sodium acetate with 0.02 % H_2O_2_and washed in water after 3 min. Finally, slides were counterstained for 3 min in hematoxylin eosin (Merck), dehydrated in a graded ethanol series, and mounted. For each series of pancreas sections, one slide was stained with second antibody only as a control for endogenous peroxidase activity and nonspecific binding of the second step. A section of spleen or lymph nodes was included as a positive control.

Quantification of immunohistochemistry

The surface areas of the pancreata were assessed via a VIDAS-RT image analysis system (Kontron Elektronik GmbH/ Carl Zeiss, Weesp, the Netherlands). Pancreata were encircled by the researcher and then measurements were performed and expressed in pixels using an objective magnification of 2x. At a magnification of 2x, the size of one pixel is $1.20*10^{-4}$ mm^2. The mean values of the surfaces of the pancreatic sections for the various strains were 0.4-1.55 mm^2 at 1 day of age, 1.4-2.5 mm^2 at 1 week of age, 2.6-4.5 mm^2 at 2 weeks of age and 15-19 mm^2 at 4 weeks of age. The numbers of positive cells, counted by two different investigators, were then expressed per mm^2 of total pancreatic surface area.

Statistical analysis

Statistical significance for differences in cell numbers was determined by means of a two-ways analysis of variance (ANOVA) followed by Newman-Keuls *post-hoc* analysis. The level of significance was set as $p<0.05$. For all measurements, a total of 5 mice (one section per mouse) was analyzed per age-strain combination.

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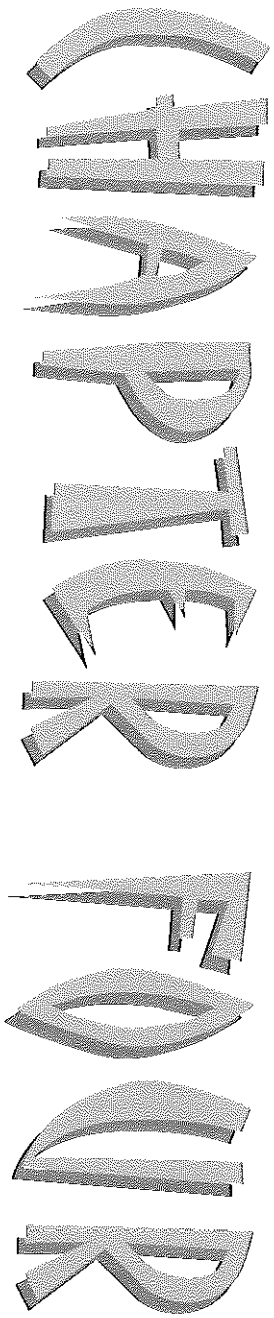
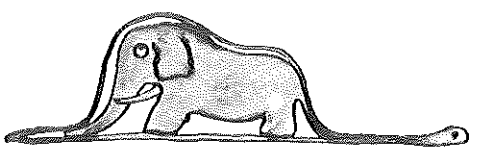


A role for islet abnormalities in the etiology of type 1 diabetes?

4.1 Islet abnormalities in the pathogenesis of type 1 diabetes: Aberrant islet development as a trigger for β cell autoimmunity?
Submitted

Chapter 4.1

Islet abnormalities in the pathogenesis of type 1 diabetes: Aberrant islet development as a trigger for β cell autoimmunity?



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Summary

NOD mice develop autoimmunity towards islets of Langerhans around weaning (i.e. 3 weeks of age). The etiology of this immune attack is unknown, but many studies have suggested the involvement of a defective regulation of the immune function (1). This explanation, however, does not take into account that the autoimmune process is strongly organ-specific. Abnormalities in immune regulation may therefore explain the predisposition to autoimmunity in general, but additional factors then determine the target of the autoimmune attack. We hypothesize that abnormalities in islet differentiation and/or function trigger the immune system towards islet autoimmunity. In this chapter, we will discuss the islet abnormalities we observed in NOD mice, together with their role in the initiation of the autoimmune response. Moreover, we will elaborate on the relevance of these observations for type 1 (insulin-dependent) diabetes mellitus (type 1 diabetes) development in humans. Finally, we will make suggestions for future research based on the results described in this thesis.

Insulitis

In order to characterize the role of islet abnormalities in the initiation of pancreatic infiltration, a thorough knowledge of the pattern of leukocyte infiltration during the initiation of insulitis and during the subsequent islet destruction is required. Therefore, we compared insulitis as observed in wild type NOD mice to rapidly destructive insulitis in NOD mice transferred with the diabetogenic T cell clone BDC2.5 and to non-destructive insulitis in NOD mice transgenic for the BDC2.5 T cell receptor (Chapter 2.1). The use of these models enabled us to study the roles of the non-lymphoid cells in a destructive and in a non-destructive insulitis, respectively. In wild-type NOD mice, the first sign of infiltration is thought to be an accumulation of $CD11c^+$ dendritic cells (DC) around the islets, from about 3-4 weeks onwards. This accumulation is present both in the destructive and in the non-destructive lesions. At the same time, ER-$MP23^+$ and MOMA-1^+ macrophages (Mϕ), normally present in the exocrine pancreas, migrate to the periphery of the islets. In lymphocyte-deficient NOD*scid* mice, the same APC infiltration takes place, albeit with lower numbers of cells (Chapter 3.1). Thus, the influx of APC occurs, in part, independent of T cells. The APC are thought to pick up islet cell antigens and migrate to the pancreas-draining lymph nodes to present these antigens to T cells. Activated islet cell-specific T cells subsequently migrate to the pancreas and home around the islets, a process called peri-insulitis. The subsequent infiltration of APC and T cells into the islets is called insulitis. In Chapter 2.1, it is shown that destructive insulitis correlates with $BM8^+$ Mϕ infiltration. Destructive insulitis will finally lead to β cell destruction and the resulting symptoms of diabetes.

β cell hyperplasia is associated with insulitis

In Chapter 2.1, we showed that the accumulation of $CD11c^+$ DC and ER-$MP23^+$ Mϕ is among the first signs of insulitis in NOD mice. Because we hypothesized that pancreatic infiltration by DC and Mϕ was due to islet abnormalities, we analyzed various endocrine parameters in NOD islets at different time points in the insulitis process. We first described an increased number of very large islets in NOD mice, which we called "mega-islets" (Chapter 3.1). These mega-islets are found at increased frequency from 10 weeks of age onwards in NOD strains, and consist mainly of β cells.

The importance of mega-islets for diabetes pathogenesis is underlined by the following observations. First, the infiltrating cells are initially predominantly situated near mega-islets. This is the case for both $CD11c^+$ DC and ER-$MP23^+$Mϕ (in NOD and NOD*scid* mice) and lymphocytes (in NOD mice) (Chapter 3.1). Second, mega-islet formation parallels diabetes incidence: mega-islet formation is delayed and reduced in non-diabetes-prone NOD males compared to diabetes-prone NOD females (Chapter 3.2). Furthermore, the enhanced diabetes incidence in orchidectomized NOD males as compared to non-manipulated NOD males is associated with an increased mega-islet formation and higher numbers of islet-infil-

trating leukocytes. This influence of orchidectomy can also be found in NOD*scid* males, indicating that mega-islet formation after orchidectomy is not the result of lymphocyte-induced damage (Chapter 3.2). Moreover, preventing the development of mega-islets by prophylactic insulin treatment also prevents the development of insulitis and diabetes in NOD mice (Chapter 3.1). In NOD*scid* mice, prophylactic insulin treatment decreases mega-islet formation and APC infiltration. These results in NOD*scid* mice indicate that the diabetes-protective effect of prophylactic insulin treatment is not obtained solely via the induction of tolerance to insulin, which is one of the important autoantigens in diabetes (2-4).

Together, these observations indicate an important link between these mega-islets, leukocyte infiltration and diabetes development. The critical role of the islet mass in insulitis initiation is also indicated by the finding that a pre-insulitic reduction of the islet mass by pancreatectomy protects NOD mice from diabetes (5,6).

β cell hyperactivity appears concomitantly with peri-insulitis

In addition to islet morphology, we analyzed islet function at the age at which the initiation of pancreatic infiltration takes place. We observed a transient hyperinsulinemia in NOD and NOD*scid* mice, which precedes mega-islet development. This hyperinsulinemia starts at 4 weeks of age, reaches its maximal value at 6-8 weeks of age, and returns to control levels at 10 weeks of age (7). The period of hyperinsulinemia is accompanied by rather low glycemia in both strains and a high insulin response to i.p. glucose injection in NOD mice. These data indicate that NOD, and to some extent, NOD*scid* β cells are hyperactive in terms of insulin secretion. The hyperinsulinemia develops concomitantly with the pancreatic APC infiltration and precedes mega-islet development in both strains.

Interestingly, pancreatic insulin contents are not different between NOD and control strains at 10 weeks of age, although NOD mice have significantly more mega-islets at this age. The observation of a low staining intensity for insulin in NOD mega-islets but not in smaller islets may explain these findings, because it suggests a low insulin content within mega-islets. It is therefore tempting to hypothesize that mega-islets, which appear to exhibit signs of a decreased β cell activity, correspond to those that have been previously hyperactive. Moreover, hyperinsulinemia in NOD mice returns to control levels at 10 weeks of age, an age at which there is not enough T cell-mediated β cell damage to explain this decrease. In addition, this decrease also occurs in NOD*scid* mice, in which there are no functional lymphocytes. Together, these observations suggest that there is a progressive and lymphocyte-independent trend toward β cell exhaustion in mice with the NOD genetic background.

Therefore, islet function appears to play an important role in the initiation of the diabetogenic process in NOD mice. It is worth noting that islet activity is influenced by many different environmental and hormonal parameters (8). Interestingly, many of these factors also influence diabetes incidence in NOD mice.

In conclusion, islets of young prediabetic NOD mice appear to be transiently hyperactive in terms of insulin secretion. This hyperactivity takes place during the post-weaning period, concomitantly with the initiation of infiltration. The transient hyperactivity is progressively followed by islet hypertrophy, resulting in an increased formation of mega-islets. These mega-islets are preferentially associated with all types of infiltrating cells. However, it is at present not clear whether these islet abnormalities are cause or consequence of the initial APC accumulation.

Potential causes of β cell hyperactivity

The next step was to analyze the putative causes of the hyperinsulinemia and subsequent mega-islet development. These islet abnormalities could have several non-mutually exclusive causes, including alterations in the (regulation of the) insulin gene, insulin-resistance, metabolic abnormalities in the prenatal period, infiltrating cells and/or their products, and hyperglucagonemia.

Mutations in the insulin gene and / or alterations in the regulation of insulin gene expression may cause differences in islet function between NOD mice and control mice. To the best of our knowledge, however, there are no data available on either the insulin gene itself, or the regulation of its expression in the NOD mouse.

Insulin-resistance is a major cause of hyperinsulinemia, particularly in type 2 diabetes. We investigated differences in insulin sensitivity between control and NOD strains, but they appeared to be only subtle (7,8).

Hyperinsulinemia in young prediabetic NOD mice can also be related to metabolic abnormalities in NOD mothers. Indeed, most NOD mothers exhibit disturbed glucose homeostasis (as indicated by an abnormal glucose tolerance and even overt diabetes) during gestation, in particular after the first or second gestation (F. Homo-Delarche, unpublished observations). As a consequence, NOD fetuses already experience high glucose levels *in utero*. Hyperinsulinemia in NOD mice starts at weaning, a period at which there is a shift from maternal milk (low in carbohydrates and high in lipids) to laboratory chow (high in carbohydrates and low in lipids). This shift triggers, even in normal mouse strains, a "wake up" of the β cells, which have been at rest because of the low glucose content of maternal milk from birth until weaning. However, the *in utero* high glucose levels in NOD mice may lead to a relatively higher insulin secretion at weaning, because of the existence of the so-called glucose-priming effect. This effect predicts that the more the β cells have been stimulated previously, the higher their insulin response to a rise in glucose levels will be (8). We therefore investigated the presence of hyperactive β cells in NOD neonates using *in situ* hybridization to the second intron of preproinsulin 2. We showed that NOD neonates have more hyperactive β cells than control neonates do, but this difference disappears within the first week of life (Chapter 3.3). Moreover, maternal insulin treatment during the last two weeks of gestation can reduce this β cell hyperactivity. This study indicates that a glucose-priming effect of NOD β cells can trigger part of the post-weaning hyperinsulinemia.

Since the initial pancreas infiltration appears concomitantly with hyperinsulinemia, we thought that infiltrating cells and their products may also be involved in the observed post-weaning islet hyperactivity. Low concentrations of cytokines, such as IL-1, are able to stimulate insulin and glucagon secretion *in vivo* and *in vitro*, while high concentrations are inhibitory and cytotoxic to β cells (9,10). In syngeneic cocultures of isolated islets of Langerhans and DC from 8-week-old mice, we found that NOD mouse DC stimulate islet insulin release, while DC from control mice do not (Chapter 3.4). Moreover, isolated NOD islets also produce more insulin *in vitro* than islets from control mice. These data indicate that β cell hyperactivity may be partially induced by DC that accumulate around the islets.

Finally, hyperinsulinemia could, partly, be the result of hyperglucagonemia (11,12). Because the first infiltrating cells are situated in the islet periphery, where mainly α cells are present, this hypothesis should also be taken into consideration, particularly because of the stimulating effects of certain cytokines on glucagon secretion. We indeed detected a slight hyperglucagonemia in NOD and NOD*scid* mice, which parallels in time the hyperinsulinemia (Chapter 3.5). Thus, a post-weaning α cell hyperactivity might also contribute to the observed β cell hyperactivity, as has already been described in animal models of type 2 diabetes (12).

In conclusion, these studies indicate that several factors might contribute to the hyperinsulinemia and, possibly, to the subsequent mega-islet development: (A) metabolic abnormalities in NOD mothers via the glucose-priming effect; (B) infiltrating cells and/or their products; and (C) hyperglucagonemia. This hyperglucagonemia, another intriguing sign of the transient islet hyperactivity observed in prediabetic NOD mice, led us to explore various parameters of α cell behavior in NOD mice at various time points from birth onwards.

Potential causes of hyperglucagonemia

We studied α cells in greater depth for several reasons. First, as already mentioned, α cells are located at the periphery of the islet, in close vicinity to the initial infiltrating cells, in contrast to β cells which form the islet core. This situation, in itself, is intriguing because the normal blood flow within the islet follows a β→α direction (13). Therefore, if the initial infiltrating leukocytes were following the normal blood flow, they would not have to pass the α cells at the islet periphery to reach the β cells, which are considered to be the main target of the autoimmune reaction. Second, while α cells appear to be spared from autoimmune destruction, there is some evidence that they themselves might be a target in human type 1 diabetes (14-17). Consequently, the close proximity of α cells to the initial infiltrating cells may affect α cell function to some extent, possible explaining the observed hyperglucagonemia. Third, glucagon secretion is tightly modulated by the gamma amino butyric acid (GABAergic) innervation, that contains the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD), a major autoantigen in type 1 diabetes. We studied the GABAergic innervation and inflammatory cells as potential causes of hyperglucagonemia in more detail.

The GABAergic innervation inhibits the secretion of glucagon and somatostatin (18). We hypothesized that a progressive loss of GABAergic innervation would result in decreased α cell inhibition, and thus hyperglucagonemia. Indeed, we showed that leukocytes surrounding GAD-containing fibers are present in the vicinity of islets with incipient peri-insulitis at 4 to 5 weeks of age. Moreover, the GABAergic innervation disappears gradually in NOD but not in control mice and, interestingly, more rapidly in diabetes-prone NOD females than in NOD males (19). Therefore, the GABAergic innervation could be among the first targets of the autoimmune process in NOD mice.

Because of the proximity of APC and α cells, we studied the effect of DC addition on glucagon secretion by isolated islets of Langerhans. Both the spontaneous glucagon release and pancreatic islet glucagon content are higher in islets isolated from NOD mice than in those of the control strain, thus confirming the *in vivo* data. However, the addition of DC does not alter significantly glucagon secretion by NOD islets. From this experiment, it can be concluded that NOD DC are not able to stimulate *in vitro* glucagon secretion. This does not, however, exclude a contribution of DC and other inflammatory cells to the observed hyperglucagonemia *in vivo*. In fact, we showed that *in vivo* administration of the APC-product IL-1 stimulates glucagon in NOD mice (Homo-Delarche, unpublished results). The absence of a stimulating effect of DC on glucagon secretion by isolated islets *in vitro* may be due to the enhanced insulin secretion that is induced by these DC. The resulting high insulin concentration in the culture medium will inhibit islet glucagon secretion.

Thus, both a loss of GABAergic innervation and, perhaps, surrounding cytokines produced by the appropriate early infiltrating cells might be involved in the observed hyperglucagonemia. However, both factors are secondary to the islet infiltrative process. They might contribute to the observed hyperglucagonemia, and via that to the observed β cell hyperactivity and islet hyperplasia, but they can not explain the etiology of the diabetogenic process. The reason for the initial islet infiltration remains unclear.

Search for abnormalities of islet development in the early postnatal period

It should be kept in mind that we observed a transient neonatal β cell hyperactivity, which clearly preceded and thus might initiate the post-weaning islet infiltration. The observation of neonatal transient β cell abnormalities suggests that post-weaning islet abnormalities might be the consequence of earlier abnormalities that spontaneously fade away after birth but reappear at time of β cell wake-up at weaning. Abnormalities of islet development could, in this scenario, account for both the post-weaning α and β cell hyperactivity. Therefore, we studied in greater depth the presence of islet abnormalities in neonates with the NOD genetic background.

At birth, the frequency of very small islets is considerably increased in both NOD and NOD*scid* mice compared to control mice (Chapter 3.5). These very small islets contain high percentages of α cells and low percentages of β cells. At the same time, i.e. before weaning,

circulating blood glucagon levels and pancreatic glucagon content are low. These data should be interpreted in the context of normal islet development, which is illustrated in Chapter 3.5. During normal development, immature islets are small and contain many α cells, which produce only low amounts of glucagon. When islet development progresses, the islet grows, mainly because of the expanding β cell fraction. The endocrine cells mature and start to produce high amounts of hormones. Therefore, the very small islets with high numbers of α cells but a low glucagon secretion and a low glucagon content, as observed in NOD and NOD*scid* mice, are reminiscent of immature islets. The presence of these immature islets suggests an increased islet neogenesis in NOD and NOD*scid* mice.

This islet neoformation may be the result of the neonatal β cell hyperactivity: a new wave of islets may be needed to later replace the already hyperactive ones that will become exhausted. Recently, we also observed disturbances in the neonatal pancreatic expression of apoptosis-associated molecules and extracellular matrix proteins, both of which are involved in islet neogenesis. Also others have obtained data suggestive of a disturbed islet development in NOD mice. Structurally normal islets of 1-month-old NOD mice contain a large percentage of $SOM^+/PDX\text{-}1^+$ cells, a cell type thought to represent an embryonic islet precursor cell (20). The presence of such cells in the islets again suggests an altered islet maturation in NOD mice. Interestingly, an altered islet maturation has also been suggested in the BB rat (21,22). Early defects in islet maturation may have important consequences for tolerance induction towards islet cells, since the expression of self-antigens during the perinatal period is critical for the establishment of self-tolerance (23,24).

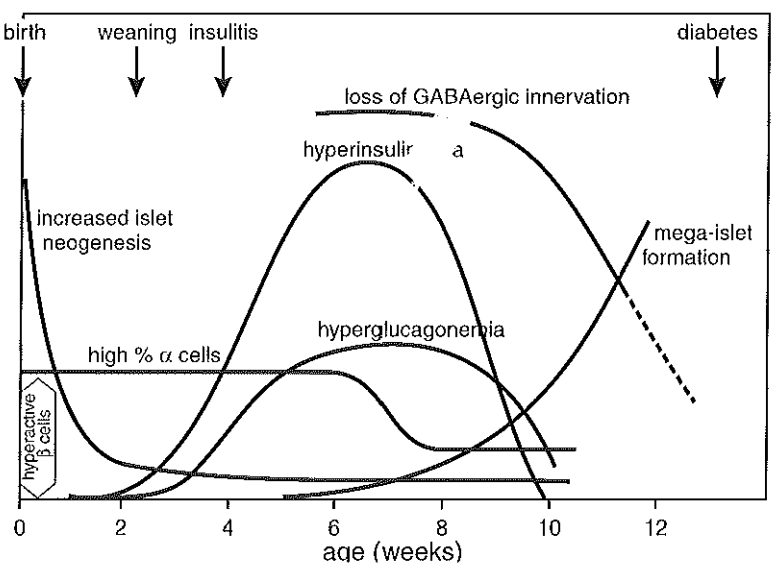

Figure 1: The development in time of the various islet abnormalities we found in NOD mice.

In summary, we investigated the presence of islet abnormalities that could trigger the diabetogenic process in NOD mice. The development in time of the various islet abnormalities we found in NOD mice is summarized in Figure 1. We postulate that an altered islet maturation occurs in NOD mice.

Since the classical insulitis in NOD mice starts after weaning, the observation of neonatal islet abnormalities seems to point to abnormalities that are inherent to the islets and not secondary to infiltrating cells. However, when examining NOD and NOD*scid* pancreases directly after birth, we observed increased numbers of APC as compared to various control strains (Chapter 3.6). Interestingly, various types of APC have been observed in locations similar to those observed in NOD mice after weaning, i.e. in peri-vascular, peri-ductular, peri-islet areas and septa. During islet neogenesis, these APC are often associated with developing islets. The APC infiltration decreases in time in all control strains and reaches very low levels in the post-weaning period, the time point at which islet development is also completed. These observations raise the question whether APC play a role in normal pancreas development. It should be realized that leukocytes have a well-recognized role during embryogenesis. For example, Mϕ play an important role in organ remodeling (25). In light of the disturbed APC function in NOD mice (Chapter 1.2), it is tempting to speculate that this disturbed function is not restricted to immune functions, but also involves the putative role for APC in pancreas development, and perhaps more particularly in islet neogenesis.

In conclusion, neonatal islet abnormalities in NOD mice (as evidenced by high numbers of hyperactive β cells accompanied by increased numbers of islets in neoformation) coincides with an early APC infiltration. Further studies are required to determine whether the disturbances in islet development are a cause or a consequence of the early leukocyte accumulation in the pancreas of NOD mice.

Etiology of diabetes in NOD mice: a scenario

In NOD mice, islet abnormalities may result in an increased susceptibility to autoimmune destruction, according to the scenario that is illustrated in Figure 2. At birth, the presence of islets with high numbers of hyperactive β cells and increased islet neogenesis is accompanied by pancreatic APC infiltration. After birth, these abnormalities progressively normalize to control strain levels. At weaning, hyperinsulinemia and hyperglucagonemia are found, concomitant with the classical APC infiltration followed by lymphocyte infiltration. The initiation of the classical insulitis correlates to the rise in blood glucose levels due to a shift from maternal milk to laboratory chow. Islet hyperactivity results in β cell exhaustion, as evidenced by the normalization of hyperinsulinemia, followed by subsequent islet hypertrophy. This hypertrophy is dependent on β cell hyperactivity, since it can be down-regulated in NOD and NOD*scid* mice by prophylactic insulin treatment.

The hypertrophy is also dependent on sex steroids, particularly on the absence of androgens, as well as on the presence of lymphocytes. The resulting mega-islets are preferentially associated with early infiltrating APC and lymphocytes.

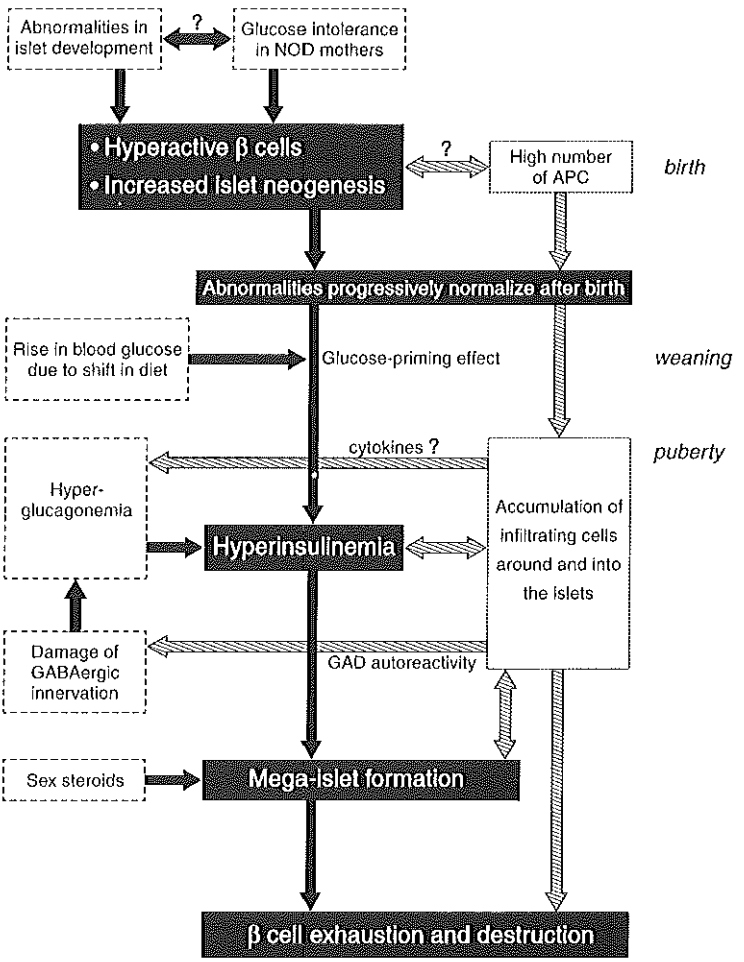

Figure 2: Etiology of autoimmune diabetes in NOD mice

Thus, a central phenomenon in this scenario is the occurrence of islet hyperactivity. There are several mechanisms via which hyperactivity increases the sensitivity of the target organ for autoimmune attack. Hyperactive endocrine cells are more prone to autoimmune reactions because of higher levels of autoantigens, adhesion and MHC molecules and also because of a higher sensitivity of the cells to cytokine-induced damage (26-29). Moreover, β cell hyperactivity can lead to the formation of neo-epitopes. One of the most interesting

candidates in this regard is proinsulin. In normal humans and animals, only very small amounts of proinsulin are released from the pancreas into the circulation. However, under sustained β cell stimulation, there is no time for the conversion of proinsulin to insulin to be completed, resulting in an increase in proinsulin secretion (30). When proinsulin-reactive T cells are deleted by the transgenic expression of proinsulin on MHC class II-bearing cells (including those in the thymus), both insulitis and diabetes are prevented (31). This indicates that proinsulin might function as a hidden cryptic epitope initiating the autoimmune-driven pancreatic infiltration in NOD mice. The subsequent lymphocyte-induced β cell damage results in higher insulin demands for the remaining β cells, leading them to become hyperactive as well. In this way, a vicious circle is started that results in the development of clinical symptoms of diabetes.

Mechanisms via which islet abnormalities can lead to a β cell specific autoimmune disease

From the scenario described above, it is evident that endocrine abnormalities are not restricted to β cells, but rather involve the entire islet. How compatible is this with the presumed β cell specificity of the autoimmune attack?

First, central tolerance induction in the thymus may be different for insulin compared to the other islet hormones. Two major differences have been found in the thymic expression of insulin compared to glucagon, somatostatin and pancreatic polypeptide (PP). The first difference is that prosomatostatin, proglucagon and proPP appear to be expressed at relatively higher levels than preproinsulin (32). The second difference is that preproinsulin is expressed on thymic DC, whereas proglucagon, prosomatostatin and proPP are expressed on thymic Mϕ (33). The presence of insulin on thymic DC is especially interesting in light of the disturbed DC function in NOD mice (Chapter 1.2). In this regard, lower levels of preproinsulin are found in NOD mouse thymus than in control thymus, whereas the levels of expression for the other pancreatic hormones are identical between the strains (32). In conclusion, the differences in thymic expression between insulin and the other islet hormones might lead to differences in tolerance induction towards these hormones.

Second, pancreas-autoimmunity in animal models of type 1 diabetes might not be as β cell specific as usually thought (34). The loss of β cells results in dramatic metabolic and clinical consequences, in contrast to the loss of other endocrine islet cells. Furthermore, as mentioned before, the specificity of both the cellular and humoral immune response may not be directed against β cells exclusively. In addition, the expression of virtually all recognized autoantigens, except proinsulin and insulin, is not restricted to β cells. Moreover, β cells are relatively more vulnerable to cytotoxins than other islet cells (35). For example, NO induces expression of the apoptosis-inducing molecule Fas on β cells only and not on other islet cells (36). In summary, autoimmunity may not be as specific for β cells as is generally assumed.

Relevance of the findings on islet abnormalities in NOD mice for human type 1 diabetes

In neonatal diabetes-prone NOD mice, we found indications for a disturbed islet development, consisting of a first wave of β cell hyperactivity and increased neogenesis associated with a slight inflammation. These abnormalities normalize in time, but a second wave of islet hyperactivity is observed after weaning, accompanied by a second wave of leukocyte infiltration, eventually resulting in β cell destruction. In humans, similar processes may play a role in the pathogenesis of type 1 diabetes.

Due to the lack of available prediabetic pancreatic tissue, it is virtually impossible to determine whether abnormalities in islet development play a role in the pathogenesis of human type 1 diabetes. Nevertheless, indirect evidence suggests that an aberrant islet development may be implicated in at least some patients. During normal human islet development, a physiological infiltration of leukocytes occurs (37-40). The composition of this infiltration is similar to that found in a child with recent-onset diabetes (39). This suggests a link between the physiological occurrence of leukocyte infiltrates during islet development and the pathophysiological infiltration of the pancreas during the initiation of autoimmunity.

Furthermore, a genetic basis may underlie a role for islet development in the etiology of human type 1 diabetes. For example, polymorphisms in the NeuroD/β2 gene, that maps to chromosome 2q32 where IDDM7 is also located (41), have been linked to human type 1 diabetes. A variant (Ala45Thr) in the NeuroD/β2 gene was found to be associated with type 1 diabetes in Japanese patients, but this association is not necessarily found in all subgroups of diabetes patients (42,43). Interestingly, this gene is thought to be essential for the morphogenesis or differentiation of insulin-producing β cells in mice (44).

The developmental abnormalities in NOD mice results in a transient prediabetic islet hyperactivity. In humans, there are also indications for islet hyperactivity preceding clinical diabetes. As already mentioned, an increased proinsulin secretion takes place in hyperactive β cells (30). Elevated basal or glucose-stimulated proinsulin and connecting (C-) peptide levels have been observed in healthy siblings of type 1 diabetes patients with no evidence of autoimmunity (45,46). These data may support a role for an aberrant β cell activity in human type 1 diabetes. Interestingly, peripheral blood T cells of individuals at risk react to a peptide that spans the B-chain of insulin and the C-peptide of proinsulin (47). This epitope can be considered a cryptic epitope, since normally it would be cleaved during conversion of proinsulin to insulin. Remarkably, this epitope bears marked similarity to a peptide of GAD65 (47). Moreover, proinsulin antibodies are detected in patients and first degree relatives (48,49). Interestingly, no association with HLA type was found for the proinsulin antibody response, in contrast to insulin autoantibodies and ICA, suggesting that the humoral response to proinsulin determinants is under separate genetic control (49).

In humans, a transient β cell hyperactivity may have a genetic basis. Genetic studies identified the *IDDM2* locus that maps to a variable number of tandem repeat (VNTR) min-

isatellites upstream the insulin gene. The short class I VNTR alleles predispose to type 1 diabetes, while the long class III have a protective effect (8). Intriguingly, several groups have reported that protective class III VNTR alleles in human adult or fetal pancreata are associated with lower insulin mRNA levels than class I alleles (50,51). It is difficult to reconcile the association of lower insulin mRNA levels in the pancreas with the finding that class III VNTR alleles are dominantly protective against type 1 diabetes. However, our data in NOD mice indicate that higher insulin mRNA levels associated with class I VNTR alleles may enhance the susceptibility of the β cells for autoimmune destruction.

Another indication for the involvement of an aberrant β cell activity in the pathogenesis of type 1 diabetes in humans is the observation that factors that influence β cell activity are also known to influence diabetes incidence. For example, prophylactic insulin treatment of subjects at risk of developing diabetes has been shown to delay the onset of overt disease (52,53). Finally, environmental factors known to trigger diabetes, such as infections, diet modification, trauma, surgery, cold, emotional stress and onset of puberty, can all affect peripheral insulin demand by creating a transient state of insulin-resistance (8,27,54).

In conclusion, in human type 1 diabetes, endocrine and immune abnormalities similar to those observed in NOD mice may play a role in the etiology and pathogenesis of the disease. Because of the heterogeneity of the human patient population and the polygenic basis of the disease, these phenomena are likely to be present in only a subgroup of the patients.

Suggestions for future research

Based on the studies described in this thesis, we suggest that an abnormal islet development, in possible relationship with a defective APC function, is involved in the initiation of islet autoimmunity in NOD mice. An extensive study, in particular during the fetal period, comparing islet development in NOD mice with that in non-diabetes-prone control strains may shed light on the postulated developmental islet abnormalities. Special emphasis should be placed on the developmental pattern of expression of the major autoantigens involved in autoimmune diabetes in NOD mice.

NOD mice exhibit developmental islet abnormalities and leukocyte defects. Moreover, alterations in the differentiation and/or function of islet cells were consistently accompanied by the presence of elevated numbers of leukocytes in the pancreas. The possible regulatory role of leukocytes in islet development and function therefore deserves further investigation, both in normal and diabetes-prone strains.

Our studies show different episodes of β cell hyperactivity from birth onwards. An aberrant regulation of the expression of the insulin gene might contribute to this phenomenon. A study of the regulation of insulin gene expression in NOD mice and humans at risk for diabetes development is required to investigate this possibility.

In conclusion, the studies described in this thesis suggest that, in type 1 diabetes, the pancreatic islet of Langerhans is not an innocent victim of the autoimmune attack. Rather, it plays an active role in the cross-talk with the immune system, both at the level of its own development and later in its destruction. In the pathogenesis of autoimmune diabetes, more (and earlier) relationships between leukocytes and pancreatic islets might occur than previously recognized. Leukocytes may regulate the activity of islets in a normal physiological way. However, for a reason still to be elucidated, APC in islets start to present autoantigens and induce an immune response. The physiological role of APC turns in a pathogenic role. Thus, the attraction between APC and islet cells during diabetes development appears to be a "fatal attraction", ultimately leading to β cell death.

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Abbreviations

AG	aminoguadine
ANOVA	analysis of variance
APC	antigen-presenting cells
β2-μ	β2-microglobulin
BB	biobreeding
CFA	complete Freund's adjuvant
C-peptide	connecting peptide
cRNA	copy ribonucleic acid
CTL	cytotoxic T lymphocyte
DC	dendritic cells
DNA	deoxyribonucleic acid
DP	diabetes-prone
DR	diabetes-resistant
DTH	delayed type hypersensitivity
EM	electron microscopy
FACS	fluorescence activated cell sorter
FasL	Fas ligand
FCS	fetal calf serum
FITC	fluoresecin-isiothiocyanate
GABA	gamma amino butyric acid
GAD	glutamic acid decarboxylase
HEV	high endothelial venule
HLA	human leukocyte antigen
ICA	islet cell antibody
ICSA	islet cell surface antibody
IFN	interferon
Ig	immunoglobulin
IL	interleukin
iNOS	inducible nitric oxide synthase
LM	light microscopy
L-NMA	N(G)-monomethyl-L-arginine
Mϕ	macrophages
MDA	malondialdehyde
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
NK	natural killer
NO	nitric oxide
NOD	nonobese diabetic
NON	nonobese non-diabetic
NOR	nonobese diabetes-resistant
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Poly I/C	polyionisinic-polycytidilic acid
PP	pancreatic polypeptide
RT-PCR	reverse transcriptase polymerase chain reaction
scid	severe combined immunodeficiency
SEM	standard error of the mean
SPF	specified pathogen free
TCR	T cell receptor
Tg	transgenic
TGF	transforming growth factor
TNF	tumor necrosis factor
TNFR	TNF receptor
TRX	thioredoxin
TUNEL	Tdt-mediated dUTP nick end labeling
VNTR	variable number of tandem repeat

Summary

Type 1 diabetes mellitus (type 1 diabetes) is the result of a T cell-mediated autoimmune destruction of the insulin-producing β cells in the pancreatic islets of Langerhans. The cause of this self-destruction is not known. Clinical symptoms of the resulting glucose intolerance occur when more than 90% of the β cells are destroyed. Hence, human type 1 diabetes patients do only present in a hospital when the autoimmune process is virtually finished. This fact disqualifies human type 1 diabetes patients as suitable subjects for the research of the initiation phase of type 1 diabetes. To study this early, decisive phase in the autoimmune process, animal models are needed. The most widely used animal model for type 1 diabetes is the nonobese diabetic (NOD) mouse. The NOD mouse spontaneously develops diabetes in a manner that closely resembles the human situation, and is thus suitable for the study of the initiation phase of type 1 diabetes.

Several abnormalities in immune function have been suggested to underlie the pathogenesis of autoimmune diabetes in NOD mice. These abnormalities do however not explain the organ-specificity of the autoimmune process. We hypothesized that abnormalities in the target organ, in addition to abnormalities in the immune system, are essential for the development of β cell-specific autoimmunity. In this thesis, we investigate the role of abnormalities in the islets of Langerhans in the etiology of autoimmune diabetes.

For this purpose, we first characterized the inflammatory infiltration of pancreatic islets with non-lymphoid cells in NOD mice, experimentally manipulated to show kinetically different pathogenic processes. We determined that the influx of $BM8^+$ Mϕ correlates with aggressiveness of the lesion. Furthermore, $ER\text{-}MP23^+$ Mϕ and $CD11c^+$ DC are cells that accumulate around the islets in the initial stages.

We then searched for abnormalities in the function and phenotype of the islets that could potentially trigger the initial infiltration with non-lymphoid cells. We found that NOD mice have an enhanced percentage of abnormally large islets, designated 'mega-islets'. Importantly, in early stages of infiltration antigen-presenting cells (APC) are preferentially associated with these mega-islets. This association suggests that mega-islet formation is an important event during diabetes development. This hypothesis is further supported by the finding that kinetics of mega-islet formation correlate with the development of disease, since it is especially pronounced in diabetes-prone NOD females and orchidectomized NOD males as compared to diabetes-resistant NOD males. In addition, preventing the formation of mega-islets by prophylactic insulin treatment also prevents the development of diabetes. Thus, mega-islet formation may be essential for diabetes to occur in NOD mice.

Several factors are shown to contribute to mega-islet formation. As already indicated, the sex difference, and thus the levels of sex steroids play a role. Preceding the generation of mega-islets, a hyperinsulinemia was found, suggesting that islet hypertrophy is the consequence of an increased insulin demand. The start of this hyperinsulinemia is associated with

the shift in diet that occurs at weaning, when maternal milk (poor in carbohydrates) is replaced by laboratory chow (rich in carbohydrates).

The dysregulated response to changing dietary conditions may be related to metabolic abnormalities that NOD mice experience during their development *in utero*. Indeed, hyperactive β cells can already be found in neonatal animals. These abnormalities normalize within one week after birth, possibly because of the neonatal diet of maternal milk. Also infiltrating cells may contribute to the hyperinsulinemia, since the development of islet infiltration by APC correlates in time with the development of hyperinsulinemia. Co-cultures of isolated islets of Langerhans and DC showed that infiltrating DC are indeed able to influence β cell activity. Another factor that contributes to prediabetic hyperinsulinemia in NOD mice is hyperglucagonemia. The association of hyperinsulinemia with hyperglucagonemia suggests a generic abnormality in islet function. Indeed, we find evidence for an abnormality in islet development in neonatal NOD mice, since the islets in neonatal NOD mice resemble immature islets. It can not be concluded, however, that these neonatal islet abnormalities are the cause of the initial islet infiltration in NOD mice. When analyzing the numbers of APC in neonatal pancreas, we found that NOD mice have higher numbers of APC in their pancreas compared to non-diabetes prone mice. Hence, the neonatal islet abnormalities may be related to an early influx of inflammatory cells.

Taken together, in this thesis, we approached the question whether abnormalities in the islets of Langerhans, in addition to abnormalities in the immune system, are involved in the development of autoimmune diabetes in NOD mice. We found several islet abnormalities, always accompanied by an inflammatory infiltrate. Thus, intrinsic islet abnormalities may indeed play a role in the etiology of NOD autoimmune diabetes. Furthermore, we showed that infiltrating leukocytes can cause islet abnormalities. Thus, a complex cross-talk between antigen-presenting cells and islets exists during development of autoimmune diabetes in NOD mice. A similar interaction may be important in the development of type 1 diabetes in humans.

Samenvatting voor niet-ingewijden

Suikerziekte, of diabetes mellitus, komt voor bij ongeveer 5% van de westerse bevolking. Suikerziekte ontstaat doordat het hormoon insuline er niet is (type 1 diabetes) of niet goed werkt (type 2 diabetes). Insuline is nodig om de brandstof van het lichaam, suiker, te kunnen gebruiken. Type 1 diabetes ontstaat meestal bij kinderen, die dan de rest van hun leven insuline moeten spuiten. Type 2 diabetes ontstaat meestal bij ouderen en kan over het algemeen met medicijnen worden behandeld. Dit proefschrift gaat over type 1 diabetes.

Type 1 diabetes is een gevolg van een vergissing van het afweersysteem. De taak van het afweersysteem is het beschermen van het lichaam tegen bacteriën en virussen. De cellen van het afweersysteem patrouilleren door het lichaam en maken bacteriën en virussen die ze tegenkomen onschadelijk. Soms maakt het afweersysteem een vergissing, en vernietigt in plaats van een bacterie of virus een stukje van het eigen lichaam. In dat geval is er sprake van een autoimmuunreactie. Autoimmuunreacties zijn de oorzaak van ziektes zoals reuma (waar het afweersysteem de gewrichten beschadigt), M.S. (waar het de zenuwen beschadigt), en type 1 diabetes. Bij type 1 diabetes vernietigt het afweersysteem de β cellen in de alvleesklier. Deze β cellen liggen samen met andere cellen in groepjes in de alvleesklier. Deze groepjes cellen worden de eilanden van Langerhans genoemd. De β cellen maken insuline; als ze vernietigd worden kan het lichaam dus geen insuline meer maken en ontstaat type 1 diabetes.

Het is niet bekend waarom het afweersysteem plotseling de β cellen aan gaat vallen. De meeste wetenschappers denken dat fouten in het afweersysteem ervoor zorgen dat dit systeem het verkeerde doelwit vernietigt. Deze theorie kan echter niet uitleggen waarom deze fouten in het afweersysteem bij sommige mensen leiden tot een afweerreactie tegen de gewrichten en dus tot reuma, terwijl bij andere mensen de β cellen worden aangevallen en dus diabetes ontstaat. Ook wij denken dat type 1 diabetes patiënten fouten in het afweersysteem hebben, maar wij gaan er bovendien van uit dat er ook fouten in de β cellen zijn. Door deze fouten in de β cellen komt het dat juist deze cellen worden vernietigd door het afweersysteem. De vraag die wordt onderzocht in dit proefschrift is: zijn er fouten in de β cellen die kunnen verklaren waarom het afweersysteem de vergissing maakt om deze cellen te vernietigen?

Deze vraag kan heel moeilijk onderzocht worden in mensen met diabetes. Dat komt doordat mensen meer dan 90% van hun β cellen kunnen missen voordat ze er iets van merken. Op het moment dat mensen ontdekken dat ze diabetes hebben, is de ziekte dus meestal al jaren ongemerkt aan de gang. Het is dus niet mogelijk om in mensen te onderzoeken hoe de ziekte is ontstaan. Onderzoek naar het ontstaan van diabetes gebeurt daarom in proefdieren. Bij dieren komt net als bij mensen ook diabetes voor. Door dieren met diabetes met elkaar te kruisen, krijg je op den duur een stam van dieren die allemaal diabetes ontwikkelen. Op deze manier is de NOD muis gemaakt, het meest gebruikte proefdier voor onderzoek naar type 1 diabetes. Deze muis ontwikkelt diabetes op een manier die erg lijkt op de ontwikkeling van type 1 diabetes bij de mens. Bovendien weten we precies op welke leeftijd de autoimmuunreactie begint in de NOD muis.

In NOD muizen kan dus de vraag worden onderzocht of fouten in de β cellen een rol spelen bij de vergissing van het afweersysteem om deze β cellen te vernietigen. We hebben op verschillende manieren deze vraag proberen te beantwoorden. Allereerst hebben we via de microscoop bekeken wat er precies gebeurt in de eilanden van Langerhans op het moment dat de autoimmuunreactie begint. We zagen dat bepaalde cellen van het afweersysteem, de zogenaamde antigeen presenterende cellen, zich verzamelden rond eilanden van Langerhans die abnormaal groot waren doordat er heel veel β cellen in zaten. Ook hebben we gekeken of de β cellen wel normaal werkten, door te bepalen hoeveel insuline de NOD muizen in hun bloed hadden op het moment dat de autoimmuunreactie begint. Het bleek dat de muizen op dit moment te veel insuline in hun bloed hadden. Het lijkt er dus op dat er inderdaad fouten zijn in de β cellen, die misschien een uitleg zijn voor de vergissing van het afweersysteem. Bovendien hebben we gevonden dat, als we deze fouten corrigeren met medicijnen, de autoimmuunreactie afneemt.

Maar deze resultaten leverden natuurlijk nieuwe vragen op. Hoe komt het eigenlijk dat de β cellen niet normaal werken in NOD muizen? We hebben gevonden dat dit van veel dingen afhangt. De moeders van NOD muizen hebben vaak zelf al suikerziekte als ze drachtig zijn, en daardoor hebben ze veel suiker in hun bloed. Dat zou gevolgen kunnen hebben voor de ontwikkeling van de NOD jongen. We hebben inderdaad gevonden dat de eilanden van Langerhans niet normaal ontwikkelen in NOD muizen. Het zou ook kunnen dat andere hormonen ervoor zorgen dat er te veel insuline wordt gemaakt. De hoeveelheid insuline in het bloed hangt sterk af van de hoeveelheid van andere hormonen, zoals de geslachtshormonen en glucagon.

We hebben gezocht naar fouten in de β cellen in de eilanden van Langerhans die zouden kunnen uitleggen waarom het afweersysteem per vergissing deze cellen vernietigt. We hebben een aantal fouten gevonden, maar we kunnen niet zeker zeggen of deze fouten ook echt de oorzaak zijn van de vernietiging door het afweersysteem. De reden is dat we ook hebben gevonden dat de autoimmuunreactie misschien veel eerder begint dan we tot nu toe dachten. We weten dus niet zeker of alle fouten die we hebben gevonden er al echt zijn voordat de autoimmuunreactie begint. Als dat niet zo is, kunnen alle gevonden afwijkingen namelijk het gevolg zijn van de autoimmuunreactie in plaats van de oorzaak.

Wij hebben geconcludeerd dat fouten in het afweersysteem en in de β cellen elkaar versterken, en er samen voor zorgen dat de antigeen presenterende cellen zich rondom de eilanden van Langerhans verzamelen. De aantrekkingskracht tussen β cellen en antigeen presenterende cellen van het afweersysteem is uiteindelijk een fatale aantrekkingskracht: de β cellen worden door de cellen van het afweersysteem vernietigd en er ontstaat suikerziekte.

Bedankt

De muziek is de moedertaal van het hart. Wat is dus een betere metafoor om mijn dankbaarheid uit te drukken dan een muzikale. Dit proefschrift is geen solo-concert, maar eerder een symfonie met een leidende melodie. Heel veel mensen hebben, ieder op hun eigen manier, een partij voor hun rekening genomen. Sommige instrumenten zijn van nature wat dominanter aanwezig dan andere, maar daarmee niet automatisch van grotere invloed. De leidende melodie krijgt juist klank en kleur door de tonen die zachtjes op de achtergrond meeklinken of die gesuggereerd worden en door de stiltes die vallen.

Hierbij wil ik alle betrokkenen bedanken voor het eindresultaat dat we samen bereikt hebben. Het met namen noemen van personen impliceert echter ook dat andere mensen, die evenzeer mee hebben gespeeld, niet genoemd worden. Daarom hoop ik dat al diegenen die ik bij het schrijven in gedachten heb gehad zich herkennen in één of meer van de hiernavolgende beschrijvingen.

Bovenal dank aan de leermeesters die ervoor hebben gezorgd dat ik in de positie kwam om op dit niveau mee te spelen. Zij hebben me geleerd dat een goed musicus iemand is die perfect speelt, maar een waardevol musicus iemand die daarnaast de muziek in al zijn facetten overziet en dit overzicht kan overdragen op het geïnteresseerde publiek. Het belangrijkste wat ze me hebben bijgebracht, is dat het leven harmonischer is als het niet alleen uit muziek bestaat.

Dank aan de componisten van het project, die ervoor zorgden dat deze symfonie geschreven werd. Ik realiseer me wel dat er van het oorspronkelijke stuk weinig overeind is gebleven, het is meer geworden tot een soort improvisatie die zeer vaag aan het oorsponkelijke thema herinnert. Maar in de muziekgeschiedenis zijn belangrijke vernieuwingen juist ontstaan als de oude regels werden losgelaten.

Dank aan de dirigenten, die de improvisaties in toom hielden en de repetities in Rotterdam en Parijs verzorgden. Bovendien lieten ze mij de verschillende interpretaties van het ruwe materiaal horen. Speciaal dank aan de dirigenten die na een slechte generale in de laatste fase de podiumvrees in toom hielden en voor voldoende vertrouwen in een goede uitvoering zorgden.

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Bovendien zijn er veel ondersteunende diensten bij een concert betrokken. Dank aan de mensen die mijn bijdrage aan het concert mogelijk hebben gemaakt, de instrumenten hebben verzorgd, de publiciteit hebben gedaan, de partijen hebben gecorrigeerd en alle verdere praktische zaken hebben geregeld.

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Het belangrijkste in de muziek staat niet in de noten. Het belangrijkste van een promotie staat dan ook niet in het proefschrift. Een promovendus leert meer van de experimenten die niet gelukt zijn dan van die die onmiddellijk succesvol waren. Net zoals een musicus alleen vooruitgang boekt door iets te spelen wat hij nog niet beheerst. Daarnaast geldt voor zowel de muziek als de wetenschap dat het geheel meer is dan de afzonderlijke delen. Juist het bereiken van dit samenspel is het verschil tussen een kakofonie en een symfonie en dus hetgene waar de speler het meeste van leert.

Een symfonie is nooit af -aangezien dat zou betekenen dat deze perfect zou zijn- maar kan altijd verbeterd worden. Het volgende concert dwingt een musicus om zijn aandacht te verleggen. In dat opzicht is een concert een momentopname, die de lading van de repetities en de kwaliteiten van de speler wellicht niet goed weerspiegelt. Maar wel een moment waar naar uitgekeken is tijdens nuttige maar vooral ook tegenvallende repetities. Daarom wil ik afsluiten met een tekst uit "Ease on down the road" uit "The Wiz", om aan terug te denken tijdens de repetities van het volgende concert:

'Cause there may be times
When you think you lost your mind -
And the steps you're takin'
Leave you three, four steps behind.
'Cause the road you're walkin'
Might be long sometime,
But just keep on steppin'
And you'll be just fine!

Judith

Curriculum vitae

De schrijfster van dit proefschrift werd geboren op 25 november 1971 in Bemmel, een dorp gelegen tussen Arnhem en Nijmegen. In diezelfde plaats volgde zij van 1984 tot 1990 het Atheneum aan de Scholengemeenschap Oost-Betuwe. In het jaar 1989-1990 werd, naast het examenjaar Atheneum, de vooropleiding dwarsfluit aan het conservatorium in Arnhem gevolgd. In 1990 besloot ze toch aan de universiteit te gaan studeren, en de keus viel op de studie medische biologie aan de Universiteit Utrecht. Tijdens deze studie was ze actief in diverse commissies op het gebied van het onderwijs. In 1993 begon ze tevens aan de studie psychologie aan de Rijksuniversiteit Leiden. In april 1995 werd het doctoraal in de medische biologie behaald, met als hoofdvakken immunologie en moleculaire neuro-endocrinologie. Op 1 mei 1995 begon ze als A.I.O. onder leiding van Prof H.A. Drexhage (Immunologie Rotterdam) en Dr. F. Homo-Delarche (Hôpital Necker, Parijs) aan het in dit proefschrift beschreven onderzoek, en besloot ze haar studie psychologie voorlopig te onderbreken. Een gedeelte van het in dit proefschrift beschreven onderzoek werd uitgevoerd in Parijs. Tijdens haar promotie heeft ze diverse vormen van onderwijs gegeven, onder andere in de histologie en in de klinische immunologie. Ook heeft ze zelf deelgenomen aan diverse cursussen, zoals stralingshygiëne niveau 3, the Oxford examination in English as a foreign language-Higher level, specialistische cursussen van de onderzoekschool Molecular Medicine en wetenschaps--theater. Bovendien was ze actief als vertegenwoordiger van het wetenschappelijke personeel in tijdelijke dienst in de staf van de vakgroep, en nam ze deel aan de organisatie van de jaarlijkse wetenschappelijke dag van de onderzoekschool Molecular Medicine. In 1996 werd de studie psychologie weer hervat, en ze koos voor de afstudeerrichting sociale en organisatiepsychologie. In augustus 1998 is ze cum laude als sociale- en organisatie-psychologe afgestudeerd op een onderzoek naar werken in multiculturele bedrijven. Sinds 1 mei 1999 is ze verbonden als postdoc aan Immunologie Rotterdam, alwaar ze werkzaam is onder leiding van Prof W. van Ewijk op een project gefinancierd door het Diabetesfonds Nederland. Het doel van dit project is het genereren van een faag antistof specifiek voor autoreactieve T cellen in type 1 diabetes.

The author of this thesis was born on November 25, 1971 in Bemmel, a village situated between Arnhem and Nijmegen. From 1984 to 1990, she attended the Oost-Betuwe Athenaeum High School in Bemmel. From 1989 to 1990, she combined High School with flute study at the music conservatory in Arnhem. In 1990, she decided to study medical biology at Utrecht University. During this period, she was a member of several committees focussing on education. She combined her study in Utrecht with the study of psychology at Leiden University from September 1993 onwards, specializing in social and organizational psychology. In April 1995, she received her Master of Science degree in medical biology, with majors in immunology and molecular neuro-endocrinology. She began her Ph.D. in May 1995 under the supervision of Prof. H.A. Drexhage (Immunology Rotterdam) and Dr. F. Homo-Delarche (Hôpital Necker, Paris). Some of the studies described in this thesis were performed at the lab in Paris. During her Ph.D.-period, she has taught diverse courses, including histology and clinical immunology. She also participated in various courses herself, including radionuclides level 3, the Oxford examination in English as a foreign language-Higher level, several advanced courses organized by the postgraduate school Molecular Medicine and scientific theater. She also was the representative of the junior scientific personnel in the staff of the department, and she participated in the organization of the annual scientific day of the postgraduate school Molecular Medicine. In August 1998, she graduated cum laude with her Master of Arts in psychology, having done research on multicultural organizations. Since May 1, 1999 she has been working as a postdoc at Immunology Rotterdam. The project she works on is subsidized by the Dutch Diabetes Fund, and is under the supervision of Prof. W. van Ewijk. The aim of this project is the generation of a single-chain phage-antibody against autoreactive T cells in type 1 diabetes mellitus.

List of publications

1. Schuil PJ, Rosmalen JGM, Graamans K, Huizing EH: Calcitonin gene-related peptide in vitro stimulation of ciliary beat in human upper respiratory cilia. Eur Arch Otorhinolaryngol 252:462-464, 1995

2. Rosmalen JGM, 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? Autoimmunity 21:28-29, 1995

3. Jansen A, Rosmalen JGM, Homo-Delarche F, Dardenne M, Drexhage HA: Effect of prophylactic insulin treatment on the number of ER-MP23^{+} macrophages in the pancreas of NOD mice. Is the prevention of diabetes based on β cell rest? J Autoimm 9:341-348, 1996

4. Rosmalen JGM, Homo-Delarche F, Dardenne M, Drexhage HA: Autoimmunity in a mouse model of type 1 diabetes mellitus is triggered by an endocrine abnormality. Immunology Letters 56:312, 1997

5. Rosmalen JGM, Leenen PJM, Katz JD, Voerman JSA, Drexhage HA: Dendritic cells in the autoimmune insulitis in NOD mouse models of diabetes. Adv Exp Med Biol 417:291-294, 1997

6. Rosmalen JGM, Homo-Delarche F, Dardenne M, Drexhage HA: The early islet infiltration of dendritic cells, macrophages and lymphocytes around NOD islets the immune cells as stimulators of β cell function and islet growth. Diabetes 47 supplement 1: A229, 1998

7. Pelegri C, Throsby M, Durant S, Rosmalen JGM, Coulaud J, Alves V, Pleau J-M, Drexhage HA, Dardenne M, Homo-Delarche F: α and β cell abnormalities in neonatal NOD mice. Diabetes & Metabolism 25 supplement 1: XXIV, 1999

8. Charre S, Rosmalen JGM, Throsby M, Drexhage HA, Leenen PJM, Alves V, Dardenne M, Homo-Delarche F: Peri-insulitis is not specific for type 1 diabetes, but a normal phenomenon during pancreas development. Diabetes & Metabolism 25 supplement 1: XXVI, 1999

9. Rosmalen JGM, Martin T, Dobbs C, Voerman JSA, Drexhage HA, Haskins K, Leenen PJM: Subsets of macrophages and dendritic cells in nonobese diabetic mouse pancreatic inflammatory infiltrates: correlation with the development of diabetes. Lab Invest 80:23-30, 2000

10. Rosmalen JGM, Homo-Delarche F, Durant S, Kap M, Leenen PJM, Drexhage HA: Islet abnormalities associated with an early influx of dendritic cells and macrophages in NOD and NOD*scid* mice. Lab Invest 80(5):in press, 2000.

11. Rosmalen JGM, Leenen PJM, Drexhage HA: Autoimmune disease: etiology and pathogenesis. In: Encyclopedia of Life Sciences, Nature Publishing Group, London, UK. In press

12. Pelegri C, Rosmalen JGM, Durant S, Throsby M, Charre S, Alves V, Coulaud J, Drexhage HA, Dardenne M, Homo-Delarche F: Islet α cell disturbances from birth onwards in mice with the nonobese diabetic genetic background. Submitted

13. Rosmalen JGM, Pigmans M, Drexhage HA, Leenen PJM, Homo-Delarche F: Sex steroid influence pancreatic islet hypertrophy and subsequent autoimmune infiltration in NOD and NOD*scid* mice. Submitted

14. Charre S, Rosmalen JGM, Pelegri C, Alves V, Leenen PJM, Drexhage HA, Homo-Delarche F: Neonatal pancreatic infiltration by dendritic cells and macrophages in mice with nonobese diabetic (NOD) or non-autoimmune genetic background. Submitted

15. Ten Bokum AMC, Rosmalen JGM, Hofland LJ, Van Hagen PM, Breemans WAP: Tissue distribution of octreotide binding sites in normal and autoimmune-prone mouse strains. Submitted

16. Throsby M, Rosmalen JGM, Pleau J-M, Coulaud J, Homo-Delarche F: Increased β cell activity in neonatal nonobese diabetic mice: in situ hybridization analysis of preproinsulin transcriptional levels. Submitted

17. Rosmalen JGM, Leenen PJM, Drexhage HA, Homo-Delarche F: Islet abnormalities in the pathogenesis of type 1 diabetes: aberrant islet development as a trigger for β cell autoimmunity? Submitted

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19. Rosmalen JGM, Van Ewijk W, Drexhage HA, Leenen PJM: T cell education during diabetes development in humans, BB rats and NOD mice: teachers and students. Submitted