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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?

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

Key words: NOD, diabetes, mega-islets, macrophages, insulin-prophylaxis

Introduction

Insulin-dependent diabetes mellitus (IDDM) is currently believed to be an autoimmune disorder which causes complete destruction of the insulin-producing cells, the β-cells of the islets of Langerhans [1–3]. The natural history of IDDM or type 1 diabetes indicates a prolonged period termed 'pre-diabetes' with no evidence of glucose intolerance, while an active process, slowly destroying β-cells, is underway. The clinical symptoms of hyperglycaemia are recognized when the β-cell mass has been reduced below 80% of the original [2, 3]. Insulin administration at this time not only corrects the hyperglycaemia, but is also associated with the recovery of endogenous insulin production, which is called the 'honeymoon phase' of IDDM [4]: the pancreatic function may therefore improve when the β-cells are relieved of hyperglycaemic stress. However, such remissions are usually not permanent. More recently, the improvement of β-cell function has been confirmed in a randomized trial of intensive insulin therapy in newly diagnosed IDDM patients,

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using a closed-loop glucose instrument, the Biostator, during the induction phase of the treatment [5]. Moreover, recurrent exposure to the Biostator was shown to decrease ICA and IAA levels [6], suggesting that the autoimmune process may cease before total β-cell destruction has occurred.

Long-term goals of type 1 diabetes research have been to develop strategies for early identification and prevention of IDDM. It has been shown that prophylactic insulin treatment of subjects at high risk of developing IDDM significantly delayed the onset of diabetes [7, 8]. The same improvement was observed in different animal models of type 1 diabetes, either induced by neonatal streptozotocin [9], or the spontaneous models, the BB rat [10–12] and the NOD mouse [13]. In both the BB rat and the NOD mouse prophylactic insulin treatment not only prevented spontaneous disease, but transfer of diabetes to young pre-diabetic animals was also inhibited [14, 15].

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

There is at present increasing evidence for a role of macrophages at early points in the disease process, as demonstrated by several approaches in the various models [19–21]. In the NOD mouse, we reported that insulitis is preceded by enlargement of the perivascular area and endothelial swelling of the islet vasculature, in combination with an accumulation of macrophages, more specifically ER-MP23$^+$, MHC-class II$^+$ macrophages with dendritic morphology, and MOMA1$^+$ macrophages [22–24]. Both cell types were the first to accumulate around the islets, before any lymphocyte infiltration. Moreover, high numbers of ER-MP23$^+$ dendritic shaped cells were also detected in the exocrine pancreas before and during the insulitis process in NOD mice. It is likely that these macrophages play a role in the presentation of islet cell antigen to T-cells. After initial accumulation of APCs around the islets, the classical lymphocytic para/peri-insulitis developed.

We thus hypothesized that if prophylactic insulin treatment induces β-cell rest, resulting in a reduction of autoantigen expression [18, 25] and release of pro-inflammatory factors from the β-cells, lower numbers of macrophages should be attracted around the islets at early stages of the disease, before any lymphocyte infiltration. In addition, such treatment might also have measurable effects on β-cell mass and β-cell insulin content. We therefore analysed the effects of prophylactic insulin treatment on the size of the islets of Langerhans, the early accumulation of ER-MP23$^+$ macrophages around the islets and in the exocrine pancreas and the development of para/peri-insulitis in the NOD mouse.

Materials and Methods

NOD mice and BALB/c mice

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

Insulin treatment

Thirty-three female NOD mice from the Paris colony were divided into two experimental groups: (1) 14 were treated with Protamine-Zinc-insulin (Organon, Oss, The Netherlands), from 4 to 6 weeks of age with 0.25 U insulin per day; from the 7th week onwards with 0.50 U insulin per day; (2) 19 served as controls and were treated with the same quantity of vehicle fluid (placebo). The above described treatment schedule is very similar to the protocol which induced significant reduction in diabetes incidence in the study described by Atkinson and coworkers [13]. However, we did not increase the dosage of insulin after the 10th week of age to 0.75 U insulin/day, since some mice became severely hypoglycaemic on this dosage of insulin and a few had already died in a previous experiment. All mice were weighed and assessed for hypoglycaemia at 3, 6, 9 and 13 weeks of age. Blood glucose levels were evaluated using Haemoglukotest test-tapes and quantitatively measured using colorimetry by Reflolux (Boehringer-Mannheim). Animals with glycaemia above 11 mmol were classified as overtly diabetic. We observed that prophylactic insulin treatment until 13 weeks of age did not alter the growth of the NOD females as compared to the placebo-treated group. Mice that became diabetic in each group during the time of the study were not included in immunohistochemical analysis.

Preparation of the pancreases

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

The remaining NOD females from both experimental groups were killed at 13 weeks of age, and their pancreases prepared for scoring of lymphocytic insulitis only. They were excised and fixed in Bouin's

Fluid, followed by 10% formalin, and were processed for paraffin embedding. Specimens were then sectioned (4 μm) at four noncontiguous levels (4–50 μm apart). For control, four BALB/c mice were killed at 9 weeks of age. Pancreases of these animals were also prepared for immunohistochemistry.

Lymphocytic insulitis

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

Monoclonal and polyclonal antibodies used to identify different types of macrophages and β-cells

ER-MP23 [22], used to identify MHC-class II$^+$ APCs was a rat–anti-mouse hybridoma culture supernatant cultured at our department and used undiluted. This marker was detected with horseradish peroxidase (HRP) labelled rabbit–anti-rat immunoglobulins (DAKO).

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

Immunohistochemistry

Immunochemistry was generally performed as described in detail in a previous report [22]. Briefly, the following procedure was performed. Before sectioning, microscopic slides were coated with a solution of 0.1% gelatine/0.01% chromiumalum in distilled water. Thereafter, 5 μm cryo-sections of the stored tissue specimens were cut, dried in air overnight, and fixed by either pararosaniline (ER-MP23) or acetone (α-insulin).

Before exposure to first step monoclonal or polyclonal antibodies, pancreases were preincubated for 10 min with 4% (v/v) normal rabbit serum. Without washing, first step monoclonal or polyclonal antibodies were applied and incubated for 1 h at room temperature. Thereafter sections were washed three times in PBS with 0.1% BSA for a total of 15 min. To diminish background staining, 1% of normal mouse serum was added to the HRP-labelled second step antibodies diluted in PBS with 1% BSA. This mixture was allowed to react for 10 min and was then applied to the cryosections. After 30 min of incubation at room temperature, sections were washed three times in PBS. Thereafter the HRP label was developed for 4 min by 0.05% DAB/0.02% H_2O_2 dissolved in PBS. Excess DAB was removed by running tap water. After counterstaining by haematoxylin the slides were dehydrated by an ethanol/xylene series and covered with Depex.

Quantification of ER-MP23$^+$ macrophages relative to the circumference of the islets and of their infiltration into the exocrine pancreas; measurement of the surface area of the islets, and quantification of the number of nuclei/μm^2 of islet surface area

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

For the measurement of the surface area of islets, only islets without lymphocyte accumulation at the circumference of the islet, and islets with only a few lymphocytes present at the circumference, were measured. In BALB/c mice 32 islets were measured in four animals, in the insulin- and placebo-treated NODs, 47 to 68 islets were assessed in six animals per group.

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

Statistical analysis

Comparison of the difference between the various groups of mice for the number of ER-MP23$^+$ macrophages per μm circumference of the islet, the number of ER-MP23$^+$ cells/mm^2 exocrine pancreas and the insulitis classifications of the two experimental groups were performed using the Mann–Whitney, two sample two-tailed signed rank test. Comparison of the number of nuclei per μm^2 islet surface area in the different experimental groups was performed with the two-sample, two-tailed Student's t-test.

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

Experimental group Insulitis	*n*	Insulitis classification % of islets (mean±SEM)		
		Intact islets	Para/peri-insulitis	Infiltrative
Placebo, 9 weeks	6	27.7±3.6	56.7±4.7	15.7±5.6
Insulin, 9 weeks	6	30.8±13.8	36.0±12.9	13.4±6.2
		P=0.18	P=0.43	P=0.93
Placebo, 13 weeks	13	20.3±6.9	4.1±1.1	75.6±7.0
Insulin, 13 weeks	8	45.7±8.5	11.5±5.1	42.8±5.2
		P=0.02	P=0.3	P=0.005

n=Number of mice.

Results

Severity of insulitis in insulin- and placebo-treated NODs

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

Effect of insulin treatment on islets in NODs. Comparison with islets of BALB/c mice

There was no immunohistochemical evidence for a lower insulin content of the β-cells (fainter staining) in any group, whether insulin- or placebo-treated NODs or BALB/c mice. What was remarkable was that the placebo-treated NODs—the group that developed the most severe insulitis—presented larger islets at 9 weeks of age than age-matched BALB/c mice (Figure 1). In placebo-treated NODs 51% of the islets were larger than 10 μm^2 in the plane of the section, and 11% were larger than 30 μm^2; in BALB/c mice, only 9% of the islets had a surface area in the plane of the section larger than 10 μm^2, and no islets were found to be larger than 30 μm^2 (difference statistically significant, P<0.0001, using Fisher's exact test with the cut-off point for comparing large and small islets set at 10 μm^2).

Interestingly, insulin treatment of the NODs reduced the number of islets with a surface area larger than 10 μm^2 to numbers found in the BALB/c, and the statistical difference between NODs and BALB/c mice

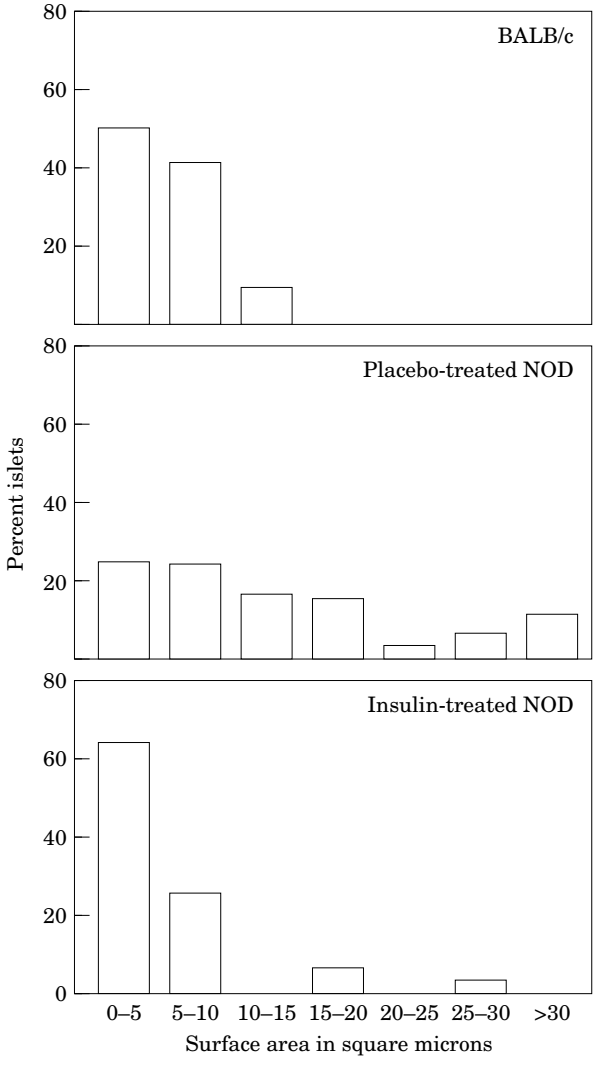

Figure 1. Distribution of islet sizes in placebo-treated NODs and insulin-treated NODs, as compared to islet sizes in BALB/c mice. The x-axis represents the islet sizes from small to large, the y-axis the percentage of islets found with a corresponding islet size. Note that the placebo-treated NODs have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

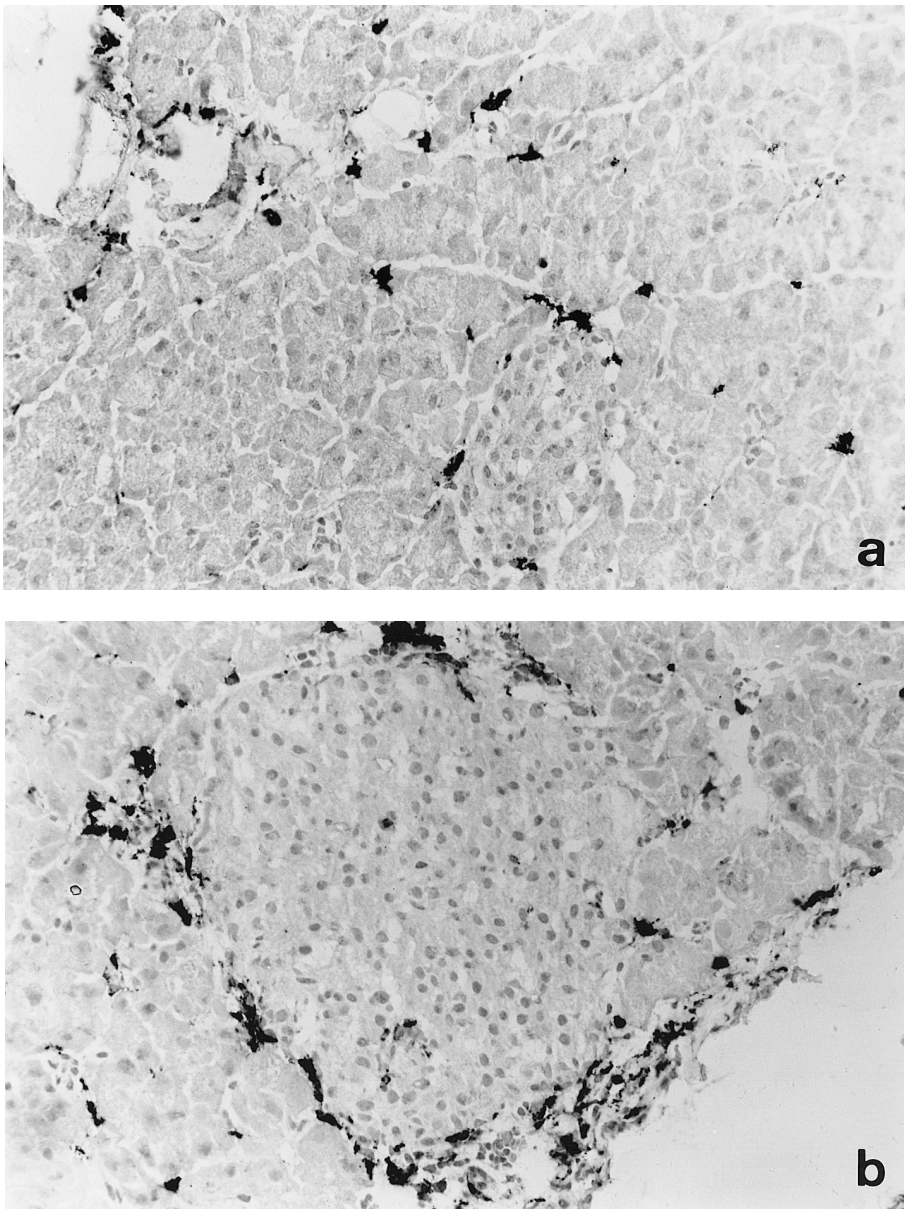

Figure 2. Islet in (a) insulin-treated female NOD of 9 weeks of age, and (b) placebo-treated female NOD of 9 weeks of age. ER-MP23 staining, magn. 250 x (in both photos). Note the considerable difference in size of the islets. Note also that the number of ER-MP23$^+$ dendritic-shaped cells (dark-coloured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

disappeared (P=0.99, see Figures 1 and 2); only 9% of the islets in insulin-treated NODs were larger than 10 μm^2 at 9 weeks of age.

Increase in islet size may be the result of β-cell hyperplasia or hypertrophia or both. The mechanism by which insulin treatment induces small-sized islets is still speculative. We therefore counted the number of nuclei per μm^2 of islet surface area. It was found that BALB/c mice have a significantly lower number of nuclei per surface area than NOD mice, whether placebo-treated or insulin-treated (P<0.05, Table 2). In spite of the reduction in islet size, insulin treatment did not alter the number of nuclei per μm^2 in islets, as compared to placebo-treated NOD mice (P=0.18).

Effect of insulin treatment on the numbers of ER-MP23$^+$ macrophages in the exocrine pancreas and around the islets in NODs as compared to numbers in BALB/c mice

In control BALB/c mice 123.1±7.1 ER-MP23$^+$ infiltrating cells per mm^2 exocrine pancreas were detectable at 9 weeks of age (Table 3). At the circumference of the islets 3.5±0.9 ER-MP23$^+$ dendritic-shaped cells were found per μm circumference. The number of ER-MP23$^+$ cells in placebo-treated NODs was significantly higher than in BALB/c mice, both in the exocrine pancreas (P=0.0006) and around islets without para/peri-insulitis (P<0.0001) at 9 weeks of age.

Table 2. *Number of β-cells per μm² of islet surface area in the different groups of mice*

Experimental groups	*n*	No. β-cells per μm² (mean±SD)	Statistical significance as compared to BALB/c mice
BALB/c	20	4.2±1.3	
Placebo-treated NOD	22	5.0±0.9	$P=0.03$
Insulin-treated NOD	22	5.3±0.9	$P=0.002$

n=Number of islets analysed.
Comparison of placebo-treated versus insulin-treated NOD: $P=0.2$.

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

Experimental group	Age	n_1	n_2	No. ER-MP23⁺ cells in the exocrine pancreas (mean±SEM/mm²)	No. ER-MP23⁺ cells at the edge of islets (mean±SEM/μm circumference)
BALB/c	9 weeks	4	16	123.1±7.1	3.5±0.9
Placebo-treated NODs	9 weeks	6	41	197.0±13.8	14.0±0.9
Insulin-treated NODs	9 weeks	6	16	156.3±8.5	7.9±1.0

n_1=Number of mice per group.
n_2=Number of islets analysed.
Main statistical significances: exocrine pancreas: placebo-treated NOD versus BALB/c, $P=0.0006$; insulin- versus placebo-treated NOD, $P=0.05$; around the islets: placebo-treated NOD versus BALB/c, $P<0.0001$; insulin- versus placebo-treated NOD, $P=0.0003$.

Prophylactic insulin treatment significantly reduced the number of ER-MP23⁺ cells in the exocrine pancreas ($P=0.05$) and around the islets ($P=0.0003$), as shown in Table 3 and Figure 2. However, insulin treatment in this study did not completely reduce the numbers of ER-MP23⁺ cells in the exocrine pancreas ($P=0.015$) and around the islets ($P=0.0035$) to levels which were found in the control BALB/c.

Discussion

This immunohistochemical study underlines the role of two different components in the pathogenesis of IDDM: first, the existence of early abnormalities of the islets of Langerhans and second, the involvement of macrophages in the progression of insulitis in the NOD mouse. In NOD mice, both components are modulated by prophylactic insulin treatment.

In this study, normal NODs exhibited large-sized islets, in contrast to BALB/c mice. NODs also showed a higher number of nuclei per μm² islet surface area, suggesting that the large-sized islets in NODs are mainly the result of β-cell hyperplasia, in the absence of a total outgrowth of the β-cells, giving rise to large islets with small β-cells. The origin of this β-cell hyperplasia in NOD mice remains to be investigated. It is essential to note that NOD-scid mice lacking lymphocytic insulitis also show large-sized islets at 6–9 weeks of age (to be published), underlining the notion that a direct effect of lymphocytes on islet cell hyperplasia can be ruled out.

Our data suggest that intrinsic islet abnormalities are related to the NOD mouse background, and precede the appearance of the autoimmune reaction, classically assessed by the appearance of lymphoid cells in the para/peri-insulitis and destructive insulitis. The functional significance of the involvement of macrophages may be analysed in the context of islet abnormalities: more macrophages and dendritic cells with an APC function are attracted to the pancreas in relation to large-sized islets (to be published).

With regard to the progression of insulitis in the NOD mouse, we confirmed the effect of prophylactic insulin treatment in lowering the severity of destructive insulitis [13, 14]. Moreover, we showed that if the effect of the treatment on the infiltrative lymphocytic insulitis, as assessed by H+E staining, was not detectable at 9 weeks of age, it became clearly visible and significant at 13 weeks of age. However, our more detailed immunohistochemical analysis detected some effects of the prophylactic insulin treatment, already visible at 9 weeks of age, consisting of a decrease in the number of accumulating antigen-presenting ER-MP23⁺ cells around the islets and in the pancreas, before any detectable lymphocytic involvement.

It is also of importance to note that prophylactic insulin treatment resulted in smaller size of the islets of Langerhans in NOD mice (comparable to the size found in BALB/c mice). The number of β-cells per μm² islet surface area was not altered by insulin treatment. Thus, though the size of the islets in insulin-treated NODs was comparable to BALB/c

islets, the number of β-cells per islet was higher, the size of the β-cells being comparable to placebo-treated NODs. This indicated that NODs may intrinsically have smaller β-cells than BALB/c mice. Insulin treatment prevents excessive β-cell hyperplasia, but permits the production of a normal amount of β-cell mass.

The reduction in islet size was associated with a lower number of infiltrative macrophages into the pancreas and around and into the islets. In addition to the role of these macrophages in initiating the autoimmune response, these cells may also be involved in the regulation of the endocrine response of the islets themselves. Also, in other organs, macrophages and dendritic cells have been found which are able to regulate the function of endocrine cells, for example, the folliculo-stellate cells in the pituitary [27] and DC-like cells and macrophages in the ovary [28] and the testis [29]. The endocrine regulatory actions of these cells are partly performed in a paracrine way, but also via cell-to-cell contact [30, 31].

Our data on an effect of prophylactic insulin treatment on islet sizes in the NOD mouse are similar to results described in BB rats [18, 32], in which transplantation of the insulin-producing RINm38 insulinoma cell line resulted in smaller islets with more quiescent β-cells having a lower expression of β-cell antigens. In fact, transient hyperactivity of the β-cells has been observed in different situations leading to IDDM [33]. This observation led to the hypothesis that the functional state of the β-cells plays an important role in the pathogenesis of type 1 diabetes. Indeed, a number of *in vitro* experiments has shown that active β-cells, producing high amounts of insulin, are more susceptible to killing by immune products than are quiescent β-cells [33, 34]. Moreover, higher β-cell activity has been associated with higher antigen expression [18, 25]. *In vivo*, the importance of a low insulin-output by β-cells is further underlined by the fact that in the BB rat, diazoxide administration, which decreases insulin secretion, reduces the incidence of diabetes [35]. Since insulin administration is well known to inhibit endogenous insulin secretion [17], essentially via an indirect neural control [36, 37], our data also probably result from β-cell rest.

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