Insulin prevents adoptive cell transfer of diabetes in the autoimmune non-obese diabetic mouse

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Summary. Early intensive insulin treatment is thought to improve subsequent Beta-cell function in Type 1 (insulin-dependent) diabetic patients. Prophylactic insulin administration also reduced diabetes incidence in diabetes-prone animals. To study the mechanisms by which these effects occur, we tested the ability of insulin therapy in the model of non-obese-diabetic mice, to prevent the penetration of committed T cells into the islets and subsequent Beta-cell destruction. Sublethally irradiated non-obese-diabetic males of 8 weeks of age were adoptively transferred with splenocytes from diabetic donors and treated with the maximum tolerable dosage of fast-acting insulin (0.5 U, twice daily) until 30 days after cell transfer. Diabetes incidence was compared to control animals injected with the same concentration of insulin diluent. After one month of treatment, the cumulative diabetes frequency was significantly less within the insulin-treated group (4 of 15, 26.6%) than in the control group (15 of 18, 83.3%; $p < 0.01$). Pancreatic histological analysis of in-

sulin-treated animals revealed a lower severity of insulitis and Beta-cell necrosis and a higher percentage of normal islets ($46.6 \pm 10\%$ vs $2.3 \pm 2\%$, $p < 0.01$), including five (33%) mice with no lesions. Immunoperoxidase staining of pancreatic sections indicated similar insulin and ganglioside staining of Beta cells from insulin-treated mice and control animals. Insulin-treated mice had comparable pancreatic insulin content to normal mice. Flow cytometry analysis of spleen cell populations indicated that insulin increased the number of Thy1,2⁺ and Lyt-2⁺ T cells. Although an effect at the Beta cell level cannot be definitely excluded, several lines of evidence suggest that insulin may influence the capacity of effector T cells to invade the islets and cause Beta-cell destruction. These effects may have potential interest for future immunointervention trials in Type 1 diabetic patients of recent onset.

Key words: NOD mouse, insulin, diabetes, prevention.

The development of animal models in which diabetes spontaneously occurs [1] as well as prospective studies of first degree relatives where immunological markers are present before the clinical onset of the disease, strongly suggest that Type 1 (insulin-dependent) diabetes mellitus is an autoimmune disease [2]. These observations have encouraged the blocking of the autoimmune Beta-cell destruction in newly-diagnosed patients. On the basis of recent protocols aimed at increasing the number of complete (insulin-free) remissions, it now becomes increasingly evident that optimization of insulin treatment at clinical onset augments the residual Beta-cell mass and increases the number of remissions [3, 4]. Several lines of evidence in animal models suggest that antigen expression of Beta cells depends on their functional state [5, 6]. Prophylactic insulin treatment of Bio/breeding (BB) rats [7, 8] and non-obese-diabetic (NOD) mice [9] during the pre-diabetes phase has been shown to prevent and/or delay the clinical onset of the disease. The precise mechanisms

which support these experimental observations are not proven. The present study was designed to examine whether insulin might be effective upon the homing in the pancreas of large amounts of autoreactive T cells transferred into naive syngeneic recipients and therefore prevent Beta-cell destruction and diabetes onset.

Materials and methods

Mice

NOD mice were bred in our facilities. The incidence of spontaneous diabetes in our colony reached 60% in females by 25 weeks whereas diabetes occurred in only 20% of males at the same period. Diagnosis of diabetes was characterized by polydipsia, weight loss, glycosuria and persistent hyperglycaemia were detected by chemstrips (Boehringer Mannheim, FRG). Diabetic NOD females served as donors of autoreactive T cells. Mice were randomized and litter matched for each study.

Cells

Splenocytes from diabetic mice were isolated in Hanks' Balanced Salt Solution (HBSS) supplemented with 2% fetal calf serum (Gibco Laboratories, Grand Island NY, USA). After numeration and viability evaluation, 10^7 cells were i.v. injected into 8-week-old irradiated NOD males (750 rads), according to the method of Wickers et al. [10] at a time when none of the NOD males had spontaneously developed the disease. In some experiments, spleen cells were first depleted of Beta cells by incubation on petri dishes coated with rabbit anti-mouse Ig (Biosys, Compiègne, France), then transferred at a concentration of 7×10^6 cells.

Protocol of insulin treatment

Insulin stock solution (Actrapid, Novo-Nordisk, Copenhagen, Denmark) was prepared with a 9% NaCl solution at a final concentration of 5 U/ml. Insulin diluent fluid was also obtained from Novo-Nordisk. These stock solutions were changed every three days to prevent insulin degradation. The day following the transfer, mice were injected s.c. twice daily with 0.5 U of insulin (equivalent approximately to 40 U per kg body weight). Control mice were simultaneously injected with the same amount of insulin diluent. This injection regimen lasted one month. The onset of glycosuria was monitored every two days starting at day 15. At day 30, all the mice were killed. Blood glucose levels (BM chemstrips, Boehringer) were monitored 18 h after the last insulin or diluent injection.

Hormonal evaluation

Circulating immunoreactive insulin concentrations were determined in experimental mice by a standard radio-immunoassay procedure (Cis International, Gisors Yvette, France) with rat insulin as standard (Novo-Nordisk). In addition, pancreata from representative animals from both groups as well as normal 8-week-old mice were extracted in 1 ml 70% ethanol-0.18 N HCl overnight at 4 °C [11], and insulin concentrations of the corresponding supernatants were determined.

Detection of insulin antibodies

Insulin antibodies were determined in the sera of experimental mice by a competitive fluid-phase radiobinding assay as previously documented [12]. Briefly, sera were stripped of insulin with charcoal-Dextran prior to incubation for 5 days at 4 °C with 7500 ng/ml of Tyr-A14 monoiodinated human insulin (specific activity 270 $\mu\text{Ci}/\mu\text{g}$ IRI, NovoBiolabs, Bagsvaerd, Denmark). Immune complexes were separated using cold polyethylene glycol. A competitive displacement with 4000 ng/ml of serum of unlabelled human insulin (Lilly, Indianapolis, Ind., USA) was performed systematically for each sample to assess specific binding to human insulin.

Histological procedures

All the mice were killed by cervical dislocation and pancreases were excised immediately and divided into two parts, one for conventional histological studies and the other for immunohistochemical procedures. For the histological studies, samples were fixed in Bouin's alcoholic solution, embedded in paraffin and semi-serial sections were stained with haematoxylin-eosin as described previously [13]. At least 25 islets were examined blind for each sample. Pancreatic islets were scored semi-quantitatively as 0 when islet cells had no visible sign of inflammation, 1 when islets had lymphocytes at the periphery, 3 when islets were completely infiltrated and 4 when islets

were atrophied. The percentages of islets of each category were then determined for each pancreas. To detect insulin-secreting cells, sections were stained with an anti-insulin guinea pig serum (Dakopatts, Santa Barbara, Calif., USA) and processed according to the peroxidase-anti-peroxidase procedure. For immunohistochemical studies with anti-ganglioside monoclonal antibodies (mAbs), frozen sections (4 μm) were fixed in cold acetone for 10 min, washed in Phosphate Buffered Saline and covered with the specific mAbs at the appropriate dilutions for 1 h.

Monoclonal antibodies

Rat IgM mAbs used for phenotypical analysis of spleen cell populations by flow cytometry were purified ascites from a panel of clones: clone 30-H12 against Thy-1.2⁺ cells [14], clone 53-6.7 against Lyt-2⁺ cells [14], and clone GK 1.5 against L3T4⁺ cells [15]. Fluoresceinated mouse mAb specific for rat kappa chain (Mark I, Biosys, Compiègne, France) was used as second reagent. FITC-rabbit anti-mouse Ig (Dakopatts, Calif., USA) was used for Beta cell analysis. Flow cytometry analysis was performed on a fluorescence-activated cell sorter (FACSCAN, Becton Dickinson, Mountain View, Calif., USA).

Mouse IgM mAbs against islet cell gangliosides were A2B5 and 3G5 (a gift from G.S.Eisenbarth, Joslin Clinic, Boston). Horseradish peroxidase conjugated anti-mouse Ig (Amersham, les Ulis, France) was used as a second reagent.

Statistical analysis

Cumulative diabetes incidences were compared by the Wilcoxon rank test. Histological scores were compared between insulin-treated and control animals using the Mann-Whitney rank test.

Results

Insulin therapy and transfer of diabetes

The maximum tolerable insulin dose compatible with survival was determined. A dose of 0.5 U of fast-acting insulin injected s.c. in three mice gave a profound and

Fig. 1. Cumulative incidence of diabetes observed during two different experiments following adoptive cell transfer. Recipients were either treated with insulin diluent (closed squares) or insulin 0.5 U twice daily (open squares). Non-transferred animals remained non-diabetic during the period of the protocol

Table 1. Characteristics of insulin- or sham-injected mice, 30 days after transfer of diabetogenic T cells. Mice were killed 18 h after the last injection

Mice	Weight (g)	Blood glucose levels (mmol/l)	Insulin concentration (mU/l)
Diabetic sham-injected	24.1 ± 0.8 <i>n</i> = 9	14.9 ± 2.2 <i>n</i> = 14	30.7 ± 5 <i>n</i> = 9
Non-diabetic insulin-treated	26.4 ± 0.8 <i>n</i> = 9	4.99 ± 0.25 <i>n</i> = 11	32.3 ± 7.5 <i>n</i> = 9
<i>p</i> value	NS	0.001	NS

Fig. 2. Cumulative incidence of diabetes during different protocols of treatment. Following adoptive cell transfer, mice were either treated twice daily with 0.9% NaCl for 28 days (*dark squares*), with insulin for 15 days then 0.9% NaCl for 13 days (*open squares*), with 0.9% NaCl for 15 days then insulin for 13 days (*dark circles*), and last with insulin for 28 days (*open circles*)

sustained drop in blood glucose levels from 5.6 ± 0.5 to 2.6 ± 0.4 mmol/l, 120 min after injection ($p = 0.002$). Blood glucose levels return to the normal range at 360 min but the levels were still significantly different from the control group at 300 min (4 ± 0.3 vs 5.7 ± 0.3 mmol/l, $p = 0.0013$). No difference was found in body weight and food intake between non-diabetic insulin and sham-injected mice. Thirty days after transfer of 10^7 splenocytes, frequency of diabetes was determined. In order to prevent any remaining effects of insulin therapy on blood glucose levels, all treatments were stopped for 18 h. The cumulative diabetes incidences from two different experiments are shown in Figure 1. The frequency of diabetes in the control group reached 83.3% ($n = 18$). By contrast, the frequency of diabetes after insulin therapy ($n = 15$) was only 26.6%. This difference was found to be statistically different ($p < 0.01$). The mean $\pm$ SD blood glucose concentration at the end of the study was 18.5 ± 1.6 mmol/l for sham-injected mice as compared with 4.75 ± 0.7 mmol/l for insulin-treated mice. Diabetic animals had a mean weight loss of 7% of their initial body weight. Determination of immunoreactive insulin by radioimmunoassay indicated that insulin treatment did not significantly modify the levels of circulating insulin levels in comparison to sham-injected mice (Table 1). No significant difference was found in the level

of antibodies to human insulin in both insulin- ($0.36 \pm 0.14\%$, $n = 10$) or sham-injected animals (0.15 ± 0.05 , $n = 9$). Analysis of hormonal content of pancreatic extracts showed lower insulin levels in diabetic or normal sham-injected mice whereas no significant difference was seen between pancreata from insulin-treated mice and normal control mice. The mean $\pm$ SEM of pancreatic insulin content from five insulin-treated mice did not differ significantly from five normal mice taken as controls (372.8 ± 41 vs 471.4 ± 66 mU/g pancreas, $p = 0.3$) while pancreata from diabetic mice with destructive insulinitis and islet atrophy had low insulin content (25.9 ± 11 mU/g pancreas, $n = 3$).

In order to determine whether protection was linked to a certain period of insulin therapy, the cumulative incidence of diabetes was compared in groups of animals transferred with 7×10^6 B cell-depleted splenocytes using different protocols. As shown in Figure 2, while mice treated with insulin for 28 days gave a complete protection, treating mice with insulin for two weeks immediately or at distance from T cell transfer did not protect animals from diabetes.

In situ studies

The results of histological analysis are shown in Figure 3. Insulin treatment protects the islets from lymphocytic infiltration and Beta-cell necrosis resulting in a higher percentage of intact islets as compared to controls ($46.6 \pm 10\%$ vs $2.3 \pm 2\%$, $p < 0.01$) and a reduced percentage of atrophied islets ($6.6 \pm 6\%$ vs $47 \pm 12\%$, $p < 0.01$) (Fig. 4a, b). Non-diabetic mice under insulin treatment ($n = 11$) did not show signs of atrophy. No difference was found in the percentages of peri-insulitis (27.8 ± 6 vs 18.6 ± 6) or insulitis (18.8 ± 7 vs $32 \pm 9\%$). In addition, pancreases from five mice (33%) which received insulin, were free of insulitis in contrast to none in the control group. Although the degree of infiltrative and destructive lesions was lower in the four mice that became diabetic despite insulin administration, these differences did not

Fig. 3. Severity of insulinitis and destructive lesions in diabetic or non-diabetic animals according to 0.9% NaCl (*dark bars*) or insulin (*open bars*) treatments. The results presented are mean percentages $\pm$ SEM of the results obtained in individual mice during two experiments. An asterisk indicates a level of significance below 0.01 using the Mann-Whitney rank test.

Fig. 4a–d. Pancreatic histology from mice transferred with committed T cells and treated with insulin diluent (**a, c**) or insulin (**b, d**) with a severe insulinitis in a sham-injected mouse (**a**) and one normal islet and one surrounded by a mild insulinitis from an insulin-injected mouse (**b**). Immunoperoxidase staining indicated comparable insulin content in residual Beta cells from control (arrowheads), (**c**) and insulin-treated animals (**d**)

reach a statistical difference. A significant difference in mean histological scores was noticed between insulin and sham-injected mice (1.46 ± 1.2 vs 3.3 ± 0.7 , $p < 0.001$).

Immunoperoxidase staining of pancreatic sections showed no change in the pattern of insulin staining of the Beta cells from both insulin- or sham-injected mice (Fig. 4c, d). Similarly, no difference between groups of experimental mice was noticed in the staining of A2B5 and 3G5 reactive gangliosides.

Effects of insulin therapy on spleen cell population

The effects of insulin therapy on T cell subsets were studied in the spleens of seven non-diabetic animals as compared to five normal and two diabetic sham-injected mice. Insulin did not affect significantly the total number of splenocytes in treated animals (103.2×10^6 vs 108.5×10^6 , $p = 0.8$). However, the phenotypical distribution of cell subsets was modified with a significant increase in the number of Thy1,2^+ T cells as indicated in Table 2. Despite the rise in the number of L3T4^+ T cells between the two groups of mice, this difference did not reach statistical significance. On the other hand, the number of Lyt-2^+ T cells was found to be significantly raised ($p < 0.05$).

Table 2. Fluorescein activated cell sorting analysis of spleen cell populations of mice without clinical diabetes, 28 days following T cell transfer. Results are mean $\pm$ SD of three different experiments

Mice	% positive			
	Thy-1,2^+	L3T4^+	Lyt-2^+	Ig^+
Sham-treated $n = 7$	32.4 ± 12	14.8 ± 10	8 ± 4	42.8 ± 10
Insulin-treated $n = 7$	45.4 ± 5	22.6 ± 9	12.3 ± 3	54.4 ± 8
<i>p</i> value	0.02	NS	< 0.05	NS

Discussion

Diabetes and Beta-cell necrosis following adoptive transfer of autoreactive T cells can be prevented by prophylactic administration of insulin to the recipients. The NOD mouse, a spontaneous model of human Type 1 diabetes, provides a unique opportunity to investigate the immune mechanisms leading to Beta-cell destruction. The role of T cell mediated immunity is emphasized by the fact that committed T cells from diabetic animals are able to invade the islets of non-diabetic syngeneic recipients and to cause the disease [10, 13]. This model of adoptive cell transfer in the NOD mouse is important, since both T cell homing in the pancreas and sequential analysis of the destructive lesions can be studied during short observation periods [13]. Godfredson et al. [7] and Like [8] with BB rats, as well as Atkinson et al. [9] with NOD mice have shown a significant reduction of diabetes incidence by prophylactic insulin treatment. These studies have also underlined that early initiation of treatment and optimal dosage were critical factors in obtaining these beneficial effects. During these protocols, the long period of treatment, exceeding 180 days, is a limitation for the study of the mechanisms involved in these preventive effects. In this study, we indicated that insulin is rapidly effective in blocking large amounts of autoreactive T cells to cause the disease. In contrast to control mice, no overt islet cell atrophy resulting from prolonged insulin therapy was found. These elements support the concept of a strong immunomodulatory effect of insulin. Although insulin therapy was associated with the conservation of a large number of insulin-producing cells sufficient to prevent hyperglycaemia in the majority of treated mice, infiltration of the islets with mononuclear cells was not completely eradicated. This suggests that insulin-therapy reduced or delayed Beta-cell destruction but did not induce a definitive state of tolerance.

In contrast to previous protocols with insulins containing zinc, a metal-ion known to decrease autoreactive T cell

function [17], we used here a fast-acting insulin and its solvent as control. Therefore, protective effects of insulin therapy can be ascribed to the insulin molecule itself. The contribution of non-specific variables like the amount of food intake could be excluded since no difference was observed before the occurrence of clinical diabetes. Current hypotheses on insulin-mediated protection are the reduction of antigen expression in Beta cells and/or modulation of effector T cells. The fact that we have demonstrated comparable immunoperoxidase staining of Beta cells for insulin both in insulin- and sham-treated mice. In addition, we have obtained comparable cytoplasmic staining of A2B5 and 3G5 reactive gangliosides in both groups of mice. Altogether, these results do not support the hypothesis that insulin therapy might place the Beta cells at rest, with a lower expression of putative autoantigens. Recent experiments have shown that glucose may induce the expression of several autoantigens, including IC2 reactive Beta-cell antigen and A2B5 reactive ganglioside [5]. Reduction of specific ganglioside expression in islet cells was observed after an insulinoma graft in BB rats [6]. However, these effects were obtained after long periods of hyperinsulinaemia which do not correspond to the conditions observed in this study. It cannot be excluded however, that low blood glucose levels in insulin-treated mice might influence the rate of Beta-cell destruction through a reduction of other specific autoantigens not explored in this study.

In fact, our observations reinforce the possibility that insulin might act upon effector T cells. Insulin has growth promoting effects on several tissues, including the thymus. Insulin therapy has been shown to restore the weight and structure of the thymus as well as the number of mature thymocytes of streptozotocin-induced diabetic rats [18]. In the present study, the number of CD3⁺ and CD8⁺ T cells increased upon insulin therapy. However, these effects could not be reproduced by treating normal NOD mice with insulin for a period of one week (data not shown). The length of insulin therapy may be critical but the effects of insulin on T cell subsets may also result from the situation of T cell reconstitution of irradiated animals. Several defects in the T cell response to alloantigens and mitogens have been demonstrated in the NOD mouse [19]. These perturbations were interpreted either as a defect in suppressor cell activation or a decrease in the number of suppressor T cells. Besides growth-promoting effects on lymphoid organs, insulin can act directly on autoreactive T cells through insulin receptors. For insulin to affect lymphocyte function, the lymphocytes must first become activated in order to express a sufficient number of insulin receptors [20]. It can be postulated that the suppressive effects on the immune response induced by insulin [21] concern mainly activated T cells.

During the recent attempts to prolong Beta-cell survival through the use of immunosuppressive agents [20], insulin requirements were used as a major indicator of success. An immunomodulatory role of insulin should also be taken into consideration for future immunointervention trials in newly-diagnosed diabetic patients. Preservation of Beta-cell function with insulin may also be

applied for short and intensive trials in prediabetic individuals with predictive markers for the disease [12, 23]

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