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Insulin-like growth factor-1 (IGF-1) protects NOD mice from insulitis and diabetes

I. BERGEROT, N. FABIEN, V. MAGUER & C. THIVOLET *INSERM U. 197 and UMR 30, Faculté de Médecine Alexis Carrel, Lyon, France*

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SUMMARY

To evaluate the effect of IGF-1 on the autoimmune process of β cell destruction, permissive non-obese diabetic (NOD) recipients were adoptively transferred with 7×10^6 autoreactive T cells from diabetic NOD mice and were administered subcutaneously 10 μg rhIGF-1, twice daily for 3 weeks. Administration of rhIGF-1 reduced the final incidence of successful transfers of diabetes observed in only 6/24 mice (25%) *versus* 12/21 (57%) in control mice. A marked reduction of insulitis during histological analysis of pancreatic glands was also observed. Mice treated with rhIGF-1 had a higher percentage of intact islets ($48{\cdot}6 \pm 12\%$ *versus* $1{\cdot}6 \pm 1{\cdot}1\%$, $P = 0{\cdot}001$) and a lower percentage of infiltrated islets. Islets from rhIGF-1-treated mice had a more intense insulin staining reflecting a higher β cell mass, but no difference was observed in the amount of insulin content of pancreatic extracts and in the amounts of mRNA transcripts for proinsulin. No difference was also observed in the titres of three islet cell antibody (ICA)-positive sera and in the pattern of A2B5 staining. Some mice developed diabetes and severe islet cell infiltration despite rhIGF-1, thus indicating that some committed T cells were still able to invade the islets and cause β cell destruction. The percentages of $CD4^+$ and $CD8^+$ T cells in the spleen of experimental mice were similar. To evaluate the effects of rhIGF-1 on cell trafficking in recipient mice, T cells from diabetic NOD Thy-1,2 mice injected into congenic NOD-N Thy-1,1 mice were monitored 3 weeks after adoptive cell transfer. The percentage of Thy-$1,2^+$ T cells was significantly reduced in the spleen ($10{\cdot}8 \pm 1{\cdot}3\%$ *versus* $17{\cdot}2 \pm 3{\cdot}9\%$, $P = 0{\cdot}004$) of rhIGF-1 treated mice in contrast to the thymus ($68{\cdot}4 \pm 7{\cdot}9\%$ *versus* $72{\cdot}87 \pm 6{\cdot}2\%$, $P = 0{\cdot}306$), suggesting that rhIGF-1 could influence T cell trafficking to the lymphoid organs. The findings that rhIGF-1 has protective effects in autoimmune diabetes opens new perspectives for future experiments as well as for preventive strategies in human type I diabetes.

Keywords IGF-1 NOD mouse diabetes autoimmunity-prevention

INTRODUCTION

The non-obese diabetic (NOD) mouse is an experimental model of spontaneous diabetes resembling human type I (insulin-dependent) diabetes, which results from the progressive islet invasion and β cell destruction by autoreactive T cells [1,2]. This spontaneous model of diabetes offers a unique opportunity to study the autoreactive T cells involved in the process of β cell destruction and to settle preventive strategies before clinical onset of the disease. The number of committed T cells in the spleens of diabetic animals [3] and the respective contribution of T cell subsets [4] can be evaluated *in vivo* during adoptive T cell transfer into non-diabetic syngeneic animals.

IGF-1, a 70-amino acid peptide structurally related to insulin, is normally considered to be a metabolic hormone which mediates many effects of growth hormone. Prophylactic insulin treatment of NOD mice during the prediabetic phase [5], as well as insulin treatment of NOD recipients of autoreactive T cells during adult T cell transfer [6] have been shown to prevent and/or delay the onset of diabetes and to reduce the severity of insulitis. Similar results have also been obtained in BB rats [7,8], another animal model of spontaneous autoimmune diabetes. Since insulin is a major antigenic component of β cells, it was not clear from these experiments whether insulin protective effects were explained by an antigen-specific unresponsiveness of the immune system, by a direct suppressive effect on T cell function, or by a direct effect on the β cells. IGF-1 is a structural analogue of insulin and acts through a type 1 IGF receptor that also shares homology with the insulin receptor. IGF-1 has also beneficial actions on the immune system [9].

Correspondence: Dr Ch. Thivolet, INSERM U. 197, Faculté de Médecine Alexis Carrel, Rue G. Paradin, 69372 Lyon Cédex 08, France.

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Therefore, the present study was undertaken to examine whether rhIGF-1 may also have protective effects in the autoimmune diabetes of NOD mice, which may explain the insulin protective effects previously observed during adoptive T cell transfer experiments.

MATERIALS AND METHODS

Mice

NOD mice were bred under standard conditions in our own facilities. The incidence of spontaneous diabetes in our colony reached 80% in females by 30 weeks, whereas diabetes occurred in only 20% of males at the same period. Congenic NOD-N Thy-1,1 mice initiated from a cross between NOD/Lt and a diabetes-resistant strain NON/Lt were obtained from E. Leiter (Bar Harbor, MN) [10]. Diagnosis of diabetes was characterized by polydipsia, weight loss, glycosuria (Urine Chemstrips; Bayer Diagnostics, Puteaux, France) and persistent hyperglycaemia (Blood Glucose Chemstrips; Lifescan, Milpitas, CA). Diabetic NOD females served as donors of autoreactive T cells. Four different experiments of adoptive cell transfer of diabetes were performed using 46 male recipients and 22 diabetic females.

Cells

Splenocytes from diabetic mice were isolated in Hanks' balanced salt solution (HBSS) and enriched T cell populations were obtained by filtration through nylon wool columns eluting 20–25% of the initial cell preparation. More than 90% of the final cell suspension was from the Thy-1,2$^+$ phenotype during flow cytometry analysis. After numeration and viability evaluation, 7×10^6 T cells were injected intravenously into 8–10-week-old irradiated NOD males (7·5 Gy) according to the method of Wicker *et al.* [3].

Protocol of treatment

Recombinant human IGF-1 (rhIGF-1) was obtained from Dr Anna Skottner (Kabi Pharmacia, Stockholm, Sweden) and aliquoted at a final concentration of 100 μg/ml. The day following adoptive cell transfer, mice were injected subcutaneously twice daily with 100 μl containing either 10 μg rhIGF-1 or saline. Recipient mice received $\approx$ 0·6 mg/kg per day of rhIGF-1 over a period of 3 weeks. The onset of glycosuria was monitored daily starting at day 15. In a separate experiment, two groups of three diabetic female mice were treated with rhIGF-1 or saline twice daily during 1 week before T cell transfer. The effects of this treatment on the capacity of diabetogenic T cells to transfer the disease were evaluated in two groups of five recipient mice.

Histological procedures and hormonal evaluation

All mice were killed by cervical dislocation, 16–18 h after the last injection. Pancreatic glands were excised and processed for conventional histological studies after fixation in Bouin's alcoholic solution. Sections (5 μm) were stained with haematoxylin–eosin as described previously [6]. The severity of insulitis was scored on at least 25 islets for each specimen, as 0 when islet cells had no visible sign of inflammation, 1 in presence of peri-insulitis, 2 when islets were mildly infiltrated (<40%), 3 when islets were completely infiltrated. The percentages of islets of each category were compared between the different groups of mice.

The number of β cells was determined by immunohistochemistry on fixed sections using a mouse anti-human insulin MoAb (Novoclone HUI 018; NovoBiolabs, Bagsvaerd, Denmark) diluted 1 : 50 and a mouse anti-human proinsulin MoAb (Novoclone HPI 005; NovoBiolabs). A FITC rabbit anti-mouse IgG (Dako, Burlingame, CA) diluted 1 : 50 was used as conjugate.

In order to examine the expression of antigen content of the islets, frozen pancreatic sections from non-diabetic animals were fixed for 2 min in acetone at room temperature, and reactivity of three type I diabetic sera containing islet cell antibodies (ICA) was assessed after preincubating the human sera with rat liver powder using a standard indirect immunofluorescence assay and a FITC-conjugated sheep anti-human IgG (Silenus, Hawthorn, Australia). Sera were titrated by serial dilutions up to 1 : 256. In separate experiments, frozen sections were incubated with a mouse anti-ganglioside MoAb A2B5 which recognizes human, rat and mouse islet cells and reacts most strongly with gangliosides that contain a trisialo sequence [11].

Pancreata from non-diabetic mice were extracted in 1 ml 70% ethanol–0·18 N HCl overnight at 4°C as previously described [6]. Free insulin concentrations of the corresponding supernatants were determined by a standard radioimmunoassay procedure (ERIA Diagnostics Pasteur, Marnes la Coquette, France).

T cell subset analysis

Following 3 weeks of treatment, spleens from experimental animals were subjected to T cell subset analysis using anti-Thy-1,2 (clone 30H12), anti-CD4 (clone GK 1,5) and anti-CD8 (clone 53–67) rat MoAbs and a FITC-conjugated anti-rat IgG κ antibody (MARK-1; Biosys, Compiègne, France). To evaluate the influence of rhIGF-1 treatment upon the homing of autoreactive T cells, the percentages of Thy-1,2$^+$ T cells injected into NOD-N Thy-1,1 recipients were determined in lymphoid organs by FACS analysis, as well as in islet infiltrates by immunohistochemical procedures on pancreatic sections.

mRNA studies

In order to study the number of mRNA transcripts for insulin in the pancreas of experimental mice, the total RNA content was precipitated in 4 M guanidine thiocyanate and 7·5 M guanidinium hydrochloride (Sigma, St Louis, MO) solutions before its extraction in chloroform-butanol (100/25, v/v). Four different concentrations of RNA ranging from 2·5 to 20 μg were hybridized on nylon membranes with ^{32}P-labelled cDNA probes for rat proinsulin (obtained from C. Dagorn, Marseille, France). After 24 h of exposure, films were analysed by densitometric scanning.

Statistical analysis

The effects of treatment on diabetes transfer were analysed using Wilcoxon test. Final incidence scores were compared using 2×2 contingency tables and χ^2 analysis. Scores of insulitis were compared using Student's t-test for unpaired samples.

RESULTS

Effects of rhIGF-1 treatment on T cell transfer of diabetes

In order to evaluate the ability of rhIGF-1 to interfere with the capacity of autoreactive T cells to transfer diabetes, recipient

Table 1. Variation in the body weight of experimental mice injected for a period of 3 weeks with rhIGF-1 or saline

Treatment	Weight (g) Day 1	Day 21	*P*
Saline	30·13 ± 0·38 ($n = 22$)	28·71 ± 0·49 ($n = 21$)	0·027
rhIGF-1	29·63 ± 0·34 ($n = 24$)	29·68 ± 0·52 ($n = 24$)	0·946

Each value represents the mean ±s.e.m. (n).

mice were treated the day following adoptive T cell transfer for an overall period of 3 weeks. The effects of rhIGF-1 on blood glucose levels were determined in normal NOD mice during separate experiments. Glucose levels dropped significantly from 122·5 ± 3·5 mg/dl to 81·5 ± 0·7 mg/dl, 30 min after a single s.c. injection of 10 μg rhIGF-1, increasing above normal values 2 h later (181 ± 19·8 mg/dl) before returning to baseline. During the treatment period, the effects of rhIGF-1 on body weight were monitored every 2 days. In contrast to saline injections, rhIGF-1 was able to maintain the body weight of recipient mice (Table 1). However, these effects were closely dependent from the onset of clinical diabetes. The occurrence of diabetes was determined in rhIGF-1-treated mice and compared with control mice during three independent experiments. Diabetes was present in 6/24 (25%) mice treated with IGF-1 and in 12/21 (57%) mice injected with saline. This difference was found significant ($\chi^2 = 4·82$, $P = 0·021$). As shown in Fig. 1, rhIGF-1 induced a significant reduction in the rate of clinical onset of the disease ($P = 0·016$).

Histological studies

The severity of insulitis was quantified and compared between the different experimental groups from two independent experiments where diabetes occurred in 4/12 (33%) mice treated with rhIGF-1 and 8/11 (72%) control mice. As shown in Fig. 2, mice treated with rhIGF-1 ($n = 12$) had a higher mean ±s.d. percentage of normal (e.g. non-infiltrated) islets (48·6 ± 12·1% *versus* 1·62 ± 1·1%, $P = 0·001$) and a lower mean ± s.d. percentage of islets with mild (15·8 ± 5·1% *versus* 31·5 ± 2·8%, $P = 0·016$) or severe insulitis (22·43 ± 8·8% *versus* 59·82 ± 6·5%, $P = 0·003$) than sham-injected mice ($n = 11$). However, no significant difference was found in the percentage of peri-insulitis (7 ± 4·5% *versus* 13·1 ± 5·8%, $P = 0·424$). Interestingly, islets from 4/12 (33%) mice which received rhIGF-1 were free from lymphocytic infiltration, in contrast to none of 11 control mice. When non-diabetic animals were compared in both groups, rhIGF-1 mice ($n = 8$) had a lower insulitis score than control mice ($n = 3$) (0·63 ± 0·8 *versus* 2·1 ± 0·4, $P = 0·02$) and a higher mean ± s.d. percentage of normal islets (67·8 ± 40% *versus* 10·2 ± 17%, $P < 0·05$). Thus, rhIGF-1 reduced both the prevalence and the intensity of insulitis.

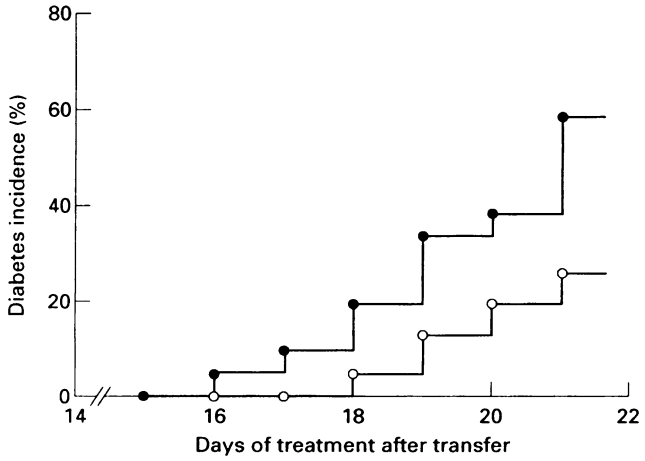

Fig. 1. Cumulative incidence of diabetes in four independent experiments following adoptive T cell transfer in 24 mice injected twice daily with 10 μg rhIGF-1 (○) and 21 control mice injected with saline (●).

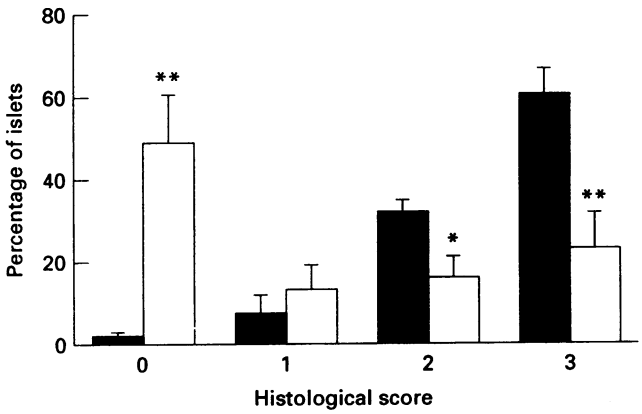

Fig. 2. Severity of insulitis and destructive lesions of recipient mice according to treatment with saline (■) or rhIGF-1 (□). Results are mean percentages ± s.e.m. from 23 individual mice from two independent experiments. $*P < 0·05$; $**P < 0·01$.

Effects of rhIGF-1 on β cells

Since the number of intact β cells was higher in rhIGF-1-treated animals, the intensity of cell fluorescence during insulin staining at the end of the treatment period was higher in rhIGF-1-treated mice, reflecting the higher β cell mass and the lower degree of islet cell infiltration (Fig. 3b,e). However, the mean ± s.d. of insulin content from pancreatic glands of non-diabetic mice was comparable between rhIGF-1-treated mice (110 ± 80 mU/g) and control mice (87 ± 32 mU/g), although the degree of insulitis was more severe in control mice. The levels of mRNA transcripts for proinsulin in non-diabetic mice during dot blot analysis were comparable in both situations. These results suggest that rhIGF-1 at the doses used in the present experiments does not modulate insulin gene expression.

In order to evaluate the immunogenicity of islet cells from experimental mice, ICA-positive sera from three recently diagnosed type I diabetic patients were titrated on frozen pancreatic sections. No difference both in pattern (Fig. 3a,d) and in titre of ICA could be noticed between mice. In addition, the pattern of A2B5 staining was comparable (Fig. 3c,f).

Effects of rhIGF-1 on T lymphocytes

No significant difference in the different T cell subsets was noticed at the end of the treatment period (Table 2). To

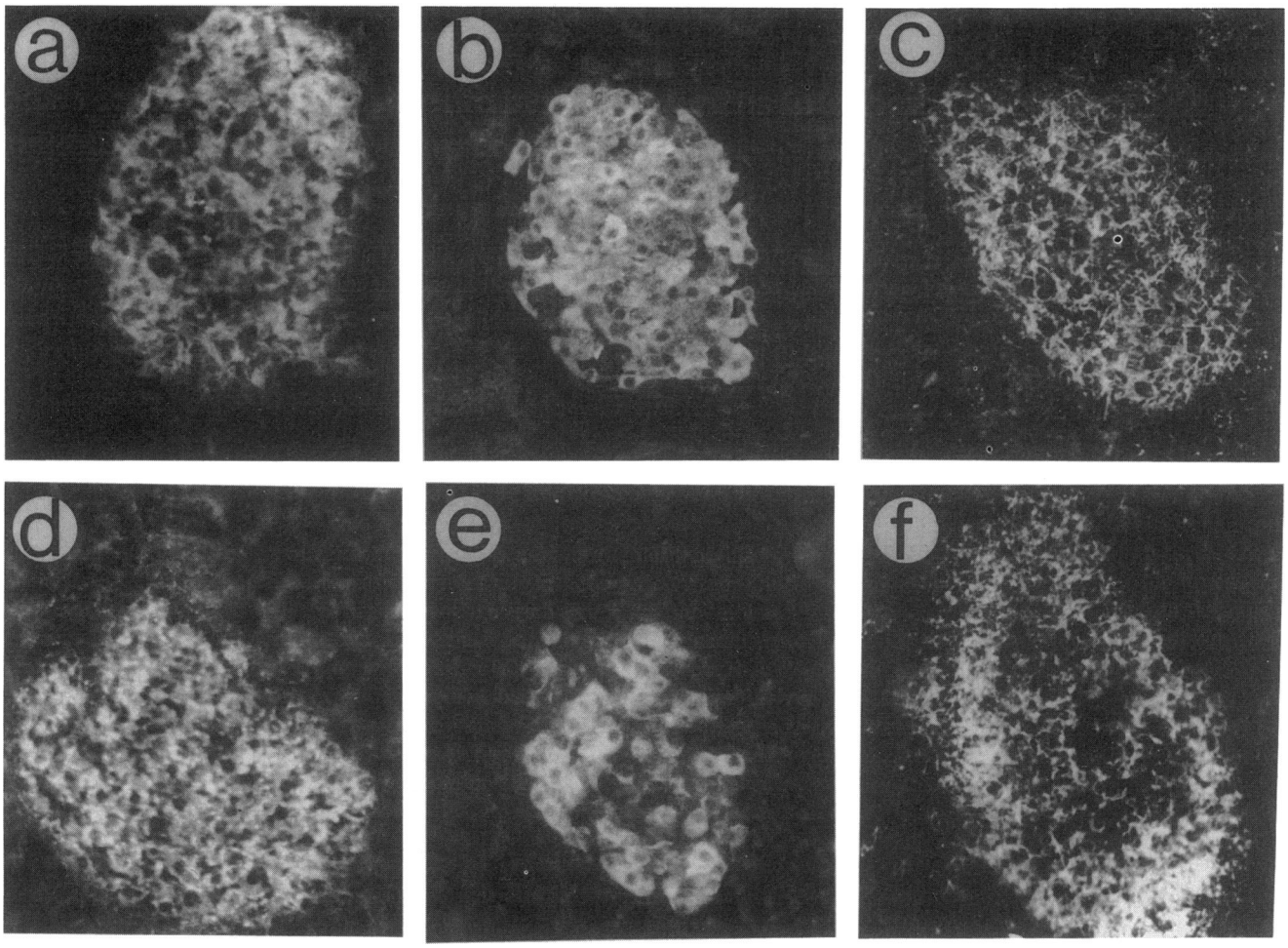

Fig. 3. Patterns of islet cell antibodies (ICA) (a,d), insulin (b,e), and A2B5 staining (c,f) of pancreatic sections from mice receiving either rhIGF-1 (a,b,c) or saline (d,e,f). IGF-1 increased the number of insulin-positive cells (b) in comparison with controls (e), reflecting a higher β cell mass.

evaluate the direct effects of rhIGF-1 on diabetogenic T cell function, we treated diabetic NOD donors with rhIGF-1 twice daily for a period of 7 days before cell transfer. Treating diabetic donors did not modify the capacity of autoreactive T cells of splenic origin to transfer the disease in irradiated male recipients. Because insulitis is a T cell phenomenon, we suspected that rhIGF-1 might interfere with the kinetics of migration of committed T cells to the pancreas. Congenic NOD-N Thy-1,1 males were adoptively transferred with T cells from diabetic NOD Thy-1,2 animals. In this experiment, diabetes occurred in 3/6 mice treated with saline and 0/6 mice treated with rhIGF-1 after 3 weeks of treatment. This protective effect

Table 2. Flow cytometry analysis of Thy-1,2$^+$, CD4$^+$ and CD8$^+$ T cells in the spleens of experimental mice treated with rhIGF-1 or saline

	Percentage of cell population		
Treatment	Thy-1,2$^+$	CD4$^+$	CD8$^+$
Saline	24·78 ± 1·05	14·98 ± 0·76	6·63 ± 0·39
rhIGF-1	21·95 ± 1·87	12·72 ± 1·48	5·19 ± 0·62
P	0·266	0·258	0·104

Results are mean ± s.e.m. of individual analysis performed in 12 different mice from each group.

Table 3. Percentages of Thy-1,2$^+$ T cells in the spleen and thymus of experimental congenic NOD-N Thy-1,1 mice 3 weeks after sublethal irradiation and adoptive transfer of 7×10^6 Thy-1,2$^+$ T cells from diabetic donors

	Thymocytes		Splenocytes	
	n ($\times 10^6$)	% of Thy-1,2$^+$ T cells	*n* ($\times 10^6$)	% of Thy-1,2$^+$ T cells
rhIGF-1 ($n = 6$)	36·8 ± 2·3	68·43 ± 3·2	60 ± 2·4	10·86 ± 0·5
Saline ($n = 6$)	35·1 ± 1·5	72·88 ± 2·5	56 ± 4·8	17·19 ± 1·6
P	0·559	0·306	0·102	0·004

Results are mean ± s.e.m. of six individual mice for each two groups.

was also associated with a decrease in severity of islet cell infiltrates, which were composed exclusively of T cells from donor origin with no recruitment of host T cells. The number of Thy-1,2$^+$ T cells in individual mice was significantly lower in the spleen of rhIGF-1-treated mice than in control mice (Table 3). No significant difference in the number of donor T cells was noticed within the thymus.

DISCUSSION

The adoptive T cell transfer model in the NOD mouse explores *in vivo* the capacity of autoreactive T cells to cause destructive lesions and ultimately type I diabetes. In the present study, we have demonstrated that rhIGF-1 is able to reduce the capacity of large amounts of committed T cells to invade NOD islets during adoptive T cell transfer. These results reproduce those previously obtained with human insulin [6] at concentrations 10 times less than those giving comparable metabolic effects in diabetic rats [12]. Despite the injection of high numbers of autoreactive T cells, rhIGF-1 was found to delay the time of onset and to reduce the maximal frequency of clinical diabetes. In addition, we present strong histological evidence for the protective effects of rhIGF-1 against massive islet cell invasion and β cell destruction.

There are distinct classes of mechanisms by which IGF-1 may prevent β cell destruction. First, the effect may be on the β cells. Specific receptors on the surface of β cells as well as local production of this growth factor have been identified [13]. Recently, enhanced IGF-1 gene expression has been shown in regenerating rat pancreas after partial pancreatectomy [14]. IGF-1 may be considered as a regulator of insulin release in view of its inhibitory effects at physiological concentrations [15]. It was interesting to note a similar insulin content in pancreatic extracts despite a lower degree of islet cell infiltration, suggesting a reduction in insulin storage. However, we were unable to find any difference in the number of mRNA transcripts for proinsulin, suggesting the absence of effects on the rate on insulin synthesis. Previous studies in NOD mice using prolonged insulin therapy [5] as well as in a model of rats injected with insulinoma cells [16] have evoked a β cell rest and a diminution in autoantigen content of the target cells. This specific point is difficult to study, since the exact target antigen of diabetogenic T lymphocytes has not been characterized. However, we were not able to show any difference in expression of the ICA antigen and A2B5 antigen in the islets of treated animals. GAD65 expression was not studied due to its low level expression in mouse islets. Beside the putative modulation of autoantigen content, rhIGF-1 may also protect the β cells through cell regeneration, protection against cytokines, or reduction of nitric oxide formation [17].

The observation of pancreatic glands free from insulitis under rhIGF-1 treatment may reflect an effect that occurs before the late activation process of infiltrating T cells by eliminating or inactivating the autoreactive T cells necessary for β cell destruction. IGF-1 may excert these effects directly on lymphoid cells, since *in vitro* suppression of the T cell response to concanavalin A (Con A) or allogeneic stimulation can be obtained in a dose-dependent manner [18]. Many actions of growth hormone on the immune system are mediated by IGF-1, which is also produced by peripheral leucocytes [19]. Recent observations suggest that activated T lymphocytes possess receptors for IGF-1 [20–22] and that IGF-1 may also influence thymic epithelial cell function *in vitro* [23] and induce thymocyte replication and differentiation in streptozocin-induced diabetic rats [24]. Mice receiving 4 mg/kg per day of rhIGF-1 had an increased spleen and thymus weight due to an increase in the number of lymphocytes in these organs, preferentially T cells from the CD4 phenotype [25]. We did not observe any difference in the number of T cells in the lymphoid organs and in the relative contribution of T cell subsets within the spleen, probably because of lower doses of rhIGF-1. Moreover, treating diabetic females with rhIGF-1 did not reduce the capacity of spleen cells to transfer the disease, suggesting that the number and degree of activation of autoreactive T cells were not modified. However, since we did not evaluate the T cell function of recipient mice, one can not exclude that IGF-1 may have some antigen-specific suppressive effects on autoreactive T cells.

The effects may be also on the mechanisms of T cell trafficking into the islets during the period of 10 days after T cell inoculation that precedes islet cell invasion [26]. T cell homing to the pancreas and endothelial–lymphocyte interactions might be regulatory events. Reconstitution of the thymus of irradiated congenic NOD-N Thy-1,1 recipients with Thy-1,2$^+$ T cells was not influenced by rhIGF-1. We observed a significant reduction in the number of T cells of donor origin noticed within the spleen. Additional experiments using pancreatic lymph nodes are in progress. A reduction in the number but not in the degree of activation of autoreactive T cells may explain why rhIGF-1-treated mice are not fully protected and diabetes onset may be only delayed.

From the present observations, rhIGF-1 should be considered a protective agent against autoimmune diabetes, which may have important clinical consequences in human type I diabetes. Our results do not reveal a simple relationship between differences in incidence of diabetes and direct β cell effects and changes in autoreactive T cell function. The importance of the β cell rest hypothesis remains to be established.

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