Repopulation of the atrophied thymus in diabetic rats by insulin-like growth factor I (streptozotocin-induced diabetes/thymocyte differentiation/flow cytometry)

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ABSTRACT  
Atrophy of the thymus is one of the consequences of severe insulin deficiency. We describe here that the weight and the architecture of the thymus of diabetic rats is restored towards normal not only by insulin but also by insulin-like growth factor I (IGF-I) treatment. In contrast to insulin, this effect of IGF-I occurs despite persisting hyperglycemia and adrenal hyperplasia. We also investigated the in vivo effect of IGF-I on replication and differentiation of thymocytes from streptozotocin-induced diabetic rats. Thymocytes from diabetic rats incorporated less [3H]thymidine than did thymocytes from healthy rats. Insulin, as well as IGF-I treatment of diabetic rats increased [3H]thymidine incorporation by thymocytes. Flow cytometry of thymocytes labeled with monoclonal antibodies revealed a decreased expression of the Thy-1 antigen in diabetic rats compared with control rats. In addition, a major deficiency of thymocytes expressing simultaneously the W3/25 and the Ox8 antigens (corresponding to immature human CD4+CD8+ thymocytes) was observed. These changes were restored towards normal by insulin as well as by IGF-I treatment. The antibody response to a T cell-dependent antigen (bovine serum albumin) was comparable in normal and diabetic rats. We conclude that IGF-I has important effects on the thymocyte number and the presence of CD4+CD8+ immature cells in the thymus of diabetic rats despite persisting hyperglycemia. However, helper T-cell function for antibody production appears to be preserved even in the severely diabetic state.

Insulin deficiency in humans is accompanied by an increased susceptibility to bacterial and mycotic infections. The reason for this is not clear, and results from different studies are inconsistent (for review, see ref. 1). Many of the immunological abnormalities are also observed in experimental diabetes in mice or rats. In streptozotocin-induced diabetic rats, the cellularity of lymphoid organs is diminished; T-cell responses (allograft reactivity and delayed T-cell hypersensitivity), B-cell responses (humoral antibodies), and phagocytic activities have been reported to be impaired (2–5). Insulin administration restores the immune response of diabetic rats toward normal. In hypophysectomized rats, depressed T- and B-cell functions as well as impaired nonspecific immune responses have been reported (6).

Hypophysectomized and diabetic rats have decreased insulin-like growth factor I (IGF-I) levels in common and, therefore, may be used to study the effects of IGF-I in vivo. In both models, low levels of IGF-I are accompanied by growth arrest (7, 8). The thymus and spleen are smaller and weigh less than in healthy rats of identical body weight (4, 6). IGF-I treatment of hypophysectomized rats and of diabetic rats leads to an increase in body weight and longitudinal bone growth and increases the cell number in primary and secondary lymphatic organs (9).

In addition to insulin deficiency, untreated diabetic rats have decreased growth hormone (GH) serum levels and GH receptor numbers and increased corticosterone levels (10–12). Similar to insulin treatment, IGF-I treatment of diabetic rats increases body weight and bone growth, but in contrast to insulin treatment, hyperglycemia, polyphagia, and polydipsia persist (13). Despite these metabolic abnormalities, the infusion of IGF-I partially restores the weight and histological appearance of the thymus of severely diabetic rats.

In this study we describe the effects of IGF-I on replication and differentiation of thymocytes and on the humoral immune response of streptozotocin-induced diabetic rats.

MATERIALS AND METHODS

Animals. Male rats of the inbred strain DA (RT1b) were purchased at the age of 5 weeks from the Hannover breeding colony (Zentralinstitut für Versuchstiere, Hannover, F.R.G.). They were kept in our animal facilities at 25°C on a 12-hr light/dark cycle and had free access to food (Haltungsdütt “A”, Kliba, Basel) and water. After an overnight fast, rats received 10 mg of diazepam per kg of body weight intraperitoneally (Valium, Hoffmann-La Roche), and streptozotocin (Zanosar, Upjohn) was injected (80 mg/kg) intravenously. Onset of diabetes was verified by glucosuria (Keto-Diabur-Test, Boehringer Mannheim). The rats were weighed daily at 0800 during the 3 weeks after induction of diabetes. Rats gaining weight were excluded from further experiments.

Insulin (Protaphan HM, Novo Industri, Bagsvaerd, Denmark) was administered subcutaneously (2 units at 8 a.m. and 4 units at 6 p.m.) with an Insulin Pen (Novo Pen II, Novo Industri).

Recombinant human IGF-I (gift from W. R. Rutter, Chiron and J. Nuechs, Ciba-Geigy) was dissolved in 0.1 M acetic acid/0.9% NaCl and filled into Alzet osmotic pumps (model 2001 or 2002, Alza). The pumps were allowed to equilibrate overnight in 5% glucose/0.9% NaCl at room temperature and were implanted subcutaneously over the thoracic spine during a short ether anesthesia. Recombinant human IGF-I was infused at a rate of 300 μg/24 hr. Alzet osmotic pumps filled with 0.1 M acetic acid/0.9% NaCl containing no hormone were implanted in the same way in diabetic control rats.

Healthy control rats were kept under identical conditions but received no streptozotocin and no further treatment. Six or 14 days after the implantation of the pumps, the rats were

Abbreviations: IGF-I, insulin-like growth factor I; GH, growth hormone; MHC, major histocompatibility complex; mAb, monoclonal antibody.

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killed by aortic puncture in general anesthesia (2 ml of Innovar-Vet per kg; Pitman-Moore, Washington Crossing, NJ). Blood glucose was immediately determined (YSI glucose analyzer). Spleen, thymus, both adrenal glands, and both epididymal fat pads were removed, dissected free of connective tissue, blotted on a filter paper, and weighed.

**Radioimmunoassays for Insulin and IGF-I.** Serum insulin was measured with a rat insulin RIA kit (NovoBiolabs).

Endogenous IGF-I and infused IGF-I were separated from serum binding proteins by chromatography on Sep-Pak C18 cartridges (Waters) according to the protocol supplied by the manufacturer and were determined by RIA with a rabbit antisemur specific for human IGF-I and cross-reactive with rat IGF-I (14). Native human IGF-I standard was a gift from R. E. Humbel (Institute of Biochemistry, University of Zürich), and rat IGF-I standard was a gift from M. Kobayashi (Fujisawa Pharmaceutical, Osaka) (15). Total IGF-I levels are given in both human and rat ng equivalents per ml of serum.

**Histology of the Thymus.** The histological sections were kindly prepared at the Institute of Children’s Pathology at the University Hospital, Zürich. All micrographs of hematoxylin/eosin-stained sections were taken at a ×40 magnification.

**[3H]Thymidine Incorporation into DNA.** After the diabetic rats had been handled for 6 days, they were killed, and cell suspensions were prepared with a stainless steel grid. The thymocyte number was determined by counting an aliquot of thymocyte suspension in an improved Neubauer cell counting chamber with or without (to obtain absolute numbers) trypan blue dye exclusion. Thymocytes from three or four rats of the same treatment group were pooled. Cells were washed twice in Ca2+- and Mg2+-free phosphate-buffered saline (PBS). Thymocytes were resuspended in 1×106 of modified Dulbecco’s medium (IMDM, GIBCO), supplemented with 100 units of penicillin per ml, 50 μg of streptomycin (GIBCO) per ml and 50 nM of 2-mercaptoethanol (GIBCO) per ml. The cell number was adjusted to 5 × 105 cells per ml. Cell viability was >95%, as assessed by trypan blue exclusion. One hundred microliters of cell suspension was distributed into 96-well flat-bottom microtiter plates (Petra Plastik, Chur, Switzerland). Cells were incubated in 200 μl of IMDM alone or IMDM supplemented with 0.5% (final dilution) fresh sterile-filtered rat serum or IMDM supplemented with 0.5% rat serum and 5 μg of concanavalin A (Con A, Pharmacia) per ml. Cultures were pulsed at the indicated time for 8 hr with 3.7 kBq of [3H]thymidine (3.22 TBq/mmol, Amersham) in 25 μl of IMDM and harvested on glass fiber paper with a cell harvester (Innotech, Wohlen, Switzerland). Filters were dried, and radioactivity was assayed in 5 ml of scintillation fluid in a β-counter (Proc. Natl. Acad. Sci. USA 87 (1990) 3691).

**Immunofluorescence and Flow Cytometry.** Mouse monoclonal antibodies (mAbs) Ox7, W3/25, Ox8, and Ox44 were purchased from Serotec. Ox7 labels all thymocytes and T lymphocytes (Thy-1.1 antigen). W3/25 and Ox8 define mutually exclusive subsets of rat T cells; W3/25 labels helper T cells. Ox44 labels a major histocompatibility complex (MHC) class I-associated antigen. It is expressed on 10–15% of thymocytes and represents those thymocytes that are on their pathway to immunocompetent T lymphocytes (16). The mouse mAb 1-11A3 recognizes MHC class II antigens of the RT1+ strain (17). A phycoerythrin (PE)-conjugated, affinity-purified F(ab′)2 rabbit anti-mouse IgG antisera (QB-AR12, Serotec) was used as the second antibody.

For immunofluorescence staining, 1 × 106 thymocytes suspended in 50 μl of PBS containing 0.2% bovine serum albumin (BSA) had been treated in a 6-day, thougout rat concentration of the appropriate mAb, washed once by centrifugation, incubated at 4°C for 1 hr with the second antibody, and washed. For two-color immunofluorescence, indirect fluorescence as described above with the mAb Ox8 was performed. The thymocytes were washed and then incubated with the directly fluorescein isothiocyanate (FITC)-labeled mAb W3/25 (SeroTec).

Cells were analyzed by flow cytometry in a fluorescence-activated cell system (FACS analyzer IFA; Becton Dickinson). Electric volume, 40° light scatter, fluorescence 1, and fluorescence 2 of each cell were determined simultaneously. Cell parameters were analyzed by using contour plots. For each experiment the percentage of cells expressing a particular marker was determined by using nonrectangular gates and was compared to the value of a negative control population. Autofluorescence and nonspecific fluorescence of the second antibody in the control samples were adjusted to the first 100 fluorescence channels to standardize conditions between different experiments. A minimum of 10,000 cells per sample were analyzed. Thymocytes from individual rats treated for 14 days were investigated.

**Immunization with BSA.** Rats were immunized with aluminum-precipitated BSA (RIA-grade, Sigma) prepared according to standard protocols (18). Antigen (500 μg) was given subcutaneously over the abdomen. The first immunization (day 0) was carried out 2 days before hormonal treatment began. A booster was given 7 days later on day 10. Rats were sacrificed on day 16. Blood was taken from the retroorbital plexus on days 3 and 6 and by aortic puncture on day 16.

**Solid-Phase RIA for Anti-BSA Antibodies.** Flexible bottom 96-well polyvinyl chloride (PVC) plates (Becton Dickinson) were coated with 5 μg of BSA in 50 μl of PBS overnight at 4°C. The remaining reactive sites on PVC were saturated with PBS containing 5% (vol/vol) fat-free milk for 2 hr at room temperature. Plates were washed twice with ice-cold PBS and incubated for 1 hr at 4°C with 25 μl of serial dilutions of rat antisera in triplicates (diluted in PBS containing fat-free milk at 0.1%). The plates were washed and incubated with 25 μl of 1:600 diluted (in 0.1% fat-free milk) goat anti-rat IgM or goat anti-rat IgG antisera (affinity-purified, Jackson ImmunReasearch) for 1 hr at 4°C. Thereafter, plates were washed and incubated for 1 hr at 4°C with 50,000 cpm of 125I-labeled protein A (Pharmacia) in 25 μl of 0.1% fat-free milk. After extensive washing, plates were dried, cut into wells, and assayed in a γ counter.

**RESULTS**

**Metabolic and Growth Parameters of Rats.** Table 1 summarizes metabolic and growth parameters as well as organ weights of the four experimental groups. The inbred rats were from a lean strain (DA); final body weight at the age of 10–12 weeks is 200–230 g. In the healthy control group, the body weight increased by 18.2 g in 14 days (at an age of 10 weeks at sacrifice), and the mean serum IGF-I level was 161 ± 7.5 ng/ml (measured against a human IGF-I standard).

The streptozotocin-induced diabetic rats, which were matched for age, lost weight and had decreased serum IGF-I levels. Blood glucose was elevated, and water and food intake were increased 4.6- and 1.6-fold, respectively. The weight of the epididymal fat pads was considerably decreased. The adrenal glands were significantly heavier than those from healthy controls. Diabetic rats treated for 14 days with insulin reached the body weight of the control animals. This means a considerable weight gain of 57.3 g in 14 days. The mean serum IGF-I level in insulin-treated diabetic rats increased to near-normal levels, and blood glucose at the time of sacrifice was normalized. Organ weights of insulin-treated diabetic rats did not differ significantly from those of the controls.

The IGF-I-infused diabetic rats remained hyperglycemic, but their body weight had increased (16.0 g in 14 days). The mean serum IGF-I level was 395 ± 40 ng/ml. Food and water intake was even higher than in saline-infused diabetic rats.
Table 1. Data from 14-day treatment of rats in four experimental groups

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>BW gain, g/14 d</th>
<th>Intake</th>
<th>Glc, mmol/liter</th>
<th>Ins, ng/ml</th>
<th>IGF-I, ng/ml</th>
<th>Organ weights, mg</th>
</tr>
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<tbody>
<tr>
<td>Rat n BW, g</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Healthy controls</td>
<td>10 204 ± 4.8</td>
<td></td>
<td>7.6 ± 0.3</td>
<td>1.05 ± 0.3</td>
<td>161 ± 62</td>
<td>153 ± 6 ± 3</td>
</tr>
<tr>
<td>D.m. Saline</td>
<td>10 125 ± 4.4</td>
<td>92 24</td>
<td>25.0 ± 1.1</td>
<td>0.36 ± 0.03</td>
<td>53 ± 24</td>
<td>58 ± 6 ± 8</td>
</tr>
<tr>
<td>Insulin</td>
<td>6 202 ± 57.3</td>
<td>33 24</td>
<td>6.8 ± 1.5</td>
<td>2.8 ± 0.03</td>
<td>395 ± 44</td>
<td>133 ± 17 ± 4</td>
</tr>
<tr>
<td>IGF-I</td>
<td>6 150 ± 16.0</td>
<td>120 30</td>
<td>20.9 ± 1.2</td>
<td>2.6 ± 0.02</td>
<td>311 ± 37</td>
<td>138 ± 25 ± 14</td>
</tr>
</tbody>
</table>

Metabolic and growth parameters and organ weights from rats of the four experimental groups (14 days of treatment)—i.e., healthy controls, saline-infused diabetic rats, insulin-treated diabetic rats, and IGF-I-infused diabetic rats. Means ± SEM are given. Food and water intake per rat were approximated from weighed daily consumption per cage. Serum IGF-I levels were measured with a human or a rat IGF-I standard and are indicated as ng equivalents per ml of serum for each standard. Organ weights were calculated per 100 g of body weight and expressed in means ± SEM. The statistical significance of the differences were determined by Student’s t test for independent samples (two-tailed). The saline-treated diabetic rats were compared with the healthy controls (levels of significance are marked by asterisks; * = P < 0.001), and the insulin- or IGF-I-treated diabetic rats were compared with the saline-infused diabetic rats (levels of significance indicated by daggers: † = P < 0.001; †† = P < 0.002). D.m., diabetes mellitus; NS, not significant; d, day(s); BW, body weight; Ins, insulin; Glc, glucose.

In diabetic rats treated with human insulin, the rat insulin level could not be determined.

Interpretation of this value should consider that infused human IGF-I is expressed in ng equivalents of a rat IGF-I standard.

The weight of the epididymal fat pads and of the adrenals was comparable to those of saline-infused diabetic rats. Their thymus weights had increased to near-normal values.

Thymus Histology. Fig. 1a depicts the thymus from a control rat. The normal lobular pattern is clearly visible. The cortex is rich in thymocytes and well delineated from the medulla. The thymus of a diabetic rat (Fig. 1b) is severely atrophic. Cortex and medulla are markedly reduced in width and depleted of thymocytes. The corticomедullary boundary is ill defined, and there is fibrosis of the medulla.

Fig. 1c shows the thymus of a diabetic rat treated with insulin for 14 days: architectural restoration is almost complete. Cortex and medulla are well delineated from each other, the pattern is again lobular, and thymocytes are numerous in the cortex, the width of which is normal.

Treatment with IGF-I for 14 days (Fig. 1d) results in a partial restoration of the histology: the thymus is much larger than in diabetic animals and has regained a distinct lobular architecture. Cortex and medulla are clearly delineated, even though they are still reduced in width. The number of thymocytes is not completely restored to normal.

[^3H]Thymidine Incorporation. In Fig. 2, thymidine uptake by thymocytes prepared from each group of rats after 6 days of treatment is shown. Thymocytes from diabetic rats incorporated significantly less[^3H]thymidine than did thymocytes from control rats. Both insulin and IGF-I treatment increased thymidine incorporation into DNA.

The response of the thymocytes to Con A was tested in further experiments. Thymidine uptake of cells in culture medium supplemented with 0.5% normal rat serum decreased with duration of the culture. Con A-stimulated thymocytes incorporated much more labeled thymidine, and there was no decrease as the culture time was prolonged to 75 hr. Thymocytes of all treatment groups reached similar maximal incorporation under Con A stimulation.

Differentiation Antigens of Thymocytes. The absolute numbers of thymocytes (× 10^6), mean ± SD per rat were 468 (± 82) for healthy control rats compared to 22 (± 3) for diabetic, 252 (± 102) for insulin-treated diabetic, and 161 (± 93) for IGF-I-infused diabetic rats (Table 2).
In diabetic rats, the number of Thy-1-expressing cells was decreased as compared with healthy controls. This diminution was partially reversed by insulin treatment. Similarly, IGF-I infusion significantly increased the number of Thy-1-positive thymocytes (Table 2).

Thymocytes expressing CD4 and CD8 antigens simultaneously were markedly reduced in the diabetic group compared with the same population of the control group. Even though large interindividual variations were observed in insulin treatment and, to a lesser extent, also IGF-I infusion clearly increased the number of CD4+/CD8+ thymocytes (Fig. 3). Although absolute numbers were decreased, the percentage of cells bearing the Ox44 mAb (defined as cells expressing an early antigen of the class I MHC)—i.e., of more mature thymocytes—was comparable in all of the experimental groups. Similarly, the percentage of thymocytes expressing the 1-11A3 antigen, which is a class II MHC antigen and a marker for activated T lymphocytes (19), did not differ significantly between the treatment groups.

**Induction of Antibodies Specific for BSA.** A secondary response to the antigen BSA was studied requiring mature helper T lymphocytes (20).

On day 6, serum titers (IgG and IgM) were between 1:3 and 1:27 with a similar distribution in all of the experimental groups. Serum titers on day 16 varied greatly within the same treatment group. The titers of anti-BSA IgG were found between 1:81 and 1:2187 for healthy controls, between 1:81 and 1:243 for saline-infused diabetic rats, between 1:9 and 1:729 for insulin-treated diabetic rats, and between 1:27 and 1:729 for IGF-I-infused diabetic rats. Interindividual variations appeared to be greater than variations between experimental groups. Intergroup analysis (Bonferroni [Dunn] T test) failed to demonstrate significant differences.

**DISCUSSION**

Diabetic rats are not only insulin deficient but also have decreased levels of GH and IGF-I in the presence of increased corticosterone levels, so that endocrine abnormalities other than insulin deficiency may play a role for their defective immune response.

To investigate the effect of IGF-I on T-cell precursors, we studied the histology and weight of the thymus as well as $[^3]H$thymidine incorporation and surface antigen expression of thymocytes from streptozotocin-induced diabetic rats that had been treated for 6 or 14 days with saline, insulin, or IGF-I. Diabetic rats had low levels of IGF-I and stopped growing. Their thymus was atrophic and depleted of thymocytes. Insulin treatment normalized the metabolic condition, increased body growth, and restored the weight and structure of the thymus. Some of these effects may be mediated by IGF-I, since insulin treatment of diabetic rats is known to normalize GH secretion and IGF-I serum levels (13, 21).
IGF-I-treated diabetic rats were still hyperglycemic but had resumed bone growth (13). Thymus weight and the number of thymocytes were increased. The histology, characteristic of a healthy thymus, was reestablished by IGF-I treatment.

Adrenalins of diabetic rats are hypertrophic and hyperactive as indicated by increased adrenal weight and by plasma corticosterone levels (12, 22). Oversecretion of the corticosterone could explain the atrophy of the thymus. However, a marked effect of IGF-I on the structure of the thymus was observed despite continuing hyperglycemia and increased adrenal activity. To simulate a chronic corticosterone over-secretion in vivo, we treated healthy rats with dexamethasone (44 µg/day) for 14 days. The thymus atrophied (thymus weights decreased to 1/10th) and was partially depleted of thymocytes, but unlike the thymus of diabetic rats, it conserved its lobular structure (not shown). Dexamethasone treatment of healthy mice was reported to have a dose-dependent profound effect on cortical thymocytes as well as on a subpopulation of medullary thymocytes and to strongly affect the cortical but not the medullary stroma (23, 24). Such thymuses are particularly deficient in immature thymocytes (24). However, the use of pharmacological doses and short-term observations render a comparison with the thymus of diabetic rats difficult.

Thymocytes of diabetic rats incorporated less [3H]thymidine into DNA than did thymocytes of healthy rats. This can be interpreted to mean that either the portion of DNA-synthesizing cells was decreased or the incorporation per cell was diminished. Insulin treatment of diabetic rats restored thymidine incorporation. The effect of IGF-I treatment was similar. This could be due to the reappearance of replicating immature thymocytes as discussed below.

The responsiveness to Con A stimulation was preserved in all of the treatment groups. Even the cells from severely diabetic rats are, therefore, functionally intact and can respond to this specific T-cell mitogen.

Some authors have reported direct depression of the immune response and lymphocyte toxicity of streptozotocin, independent of its diabetogenic actions (3, 25). The finding that thymocytes from insulin-treated streptozotocin-induced diabetic rats resemble the thymocytes from controls in every respect argues against direct streptozotocin toxicity.

Lymphopenia has been described in the peripheral blood, thymus, lymph nodes, and spleen in experimentally induced diabetes (pancreatectomy, alloxan, or streptozotocin) as well as in the genetically diabetes-prone BB rat (26–29). In the BB rat, T-lymphopenia with reduced CD4+ cells and with a major loss of CD8+ cells has been reported (29). We found two major alterations in the expression of surface antigens in thymocytes from streptozotocin-induced diabetic rats: a decrease in the expression of the Thy-1 antigen, a pan-T-cell marker, and a decrease in immature thymocytes as characterized by simultaneous expression of the CD4 and CD8 antigens. The relatively large variations within the hormone-treated diabetic groups are explained by the experimental protocol: interindividual variations within diabetic rats before treatment may amplify variations in response to the treatment. Furthermore, the duration of the treatment may play a crucial role, since the thymic stroma has to recover as well as the expression of the Thy-1 antigen and the number of CD4+/CD8- thymocytes, IGF-I proved to have an important effect on thymocyte maturation. CD4+/CD8- thymocytes represent the major source of future immunocompetent T-cells (30, 31). These IGF-I effects appear to be independent of those of insulin. However, the mechanism, which could be direct or mediated by the thymic stroma and/or by secretion of thymus-derived peptides remains to be clarified. We investigated the capacity of the different groups of rats to synthesize antibodies specific for BSA, which involves several cell lines of the immune system. Diabetic rats produced antibody titers comparable to those observed in healthy controls. Similar findings were published earlier (28, 32) and point to the remarkable reserves of the immune system.

IGF-I appears to promote cell number and structural rearrangement of the atrophic thymus in streptozotocin-induced diabetic rats. In addition, in IGF-I-infused diabetic rats, immature thymocytes, which are markedly diminished in untreated diabetic rats, reappear. This effect occurs despite persisting hyperglycemia and increased adrenal activity. Nevertheless, the in vivo processing of antigen (BSA), requiring the help of mature T lymphocytes, appears to be conserved even in the severely diabetic state.

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