# Autoimmune Diabetes Induced by the β-Cell Toxin STZ

## Immunity to the 60-kDa Heat Shock Protein and to Insulin

**Dana Elias, Haia Prigozin, Natalie Polak, Micha Rapoport, Ansgar W. Lohse, and Irun R. Cohen**

**Administered at a suitably low dose, the toxin streptozotocin (STZ) can trigger an autoimmune process leading to destruction of the β-cells of the pancreatic islets. In this study, we examined specific immunological reactions in mice before and during the development of STZ-induced autoimmune diabetes. We now report that the development of spontaneous autoantibodies to insulin can serve as a marker of susceptibility to a low dose of STZ. Susceptible male mice of the C57BL/KsJ strain manifested such anti-insulin antibodies, and resistant female mice did not. Administration of a low dose of STZ (five daily doses each of 30 mg/kg) induced transient hyperglycemia ~20–30 days later, which temporarily remitted but was followed by intractable diabetes ~2.5 months later. The diabetogenic process triggered by the low dose of STZ was associated with an increase in the level of anti-insulin antibodies bearing the Dana and Micha (DM) idiotype, later followed by the appearance of anti-idiotypic antibodies that peaked before the onset of diabetes. Antibodies and T-cells reactive to hsp60 (heat shock protein) were triggered by the low-dose STZ administration and persisted throughout the period that preceded clinical diabetes. T-cells reactive to the p277 peptide of hsp60 were also observed. Finally, active immunization to hsp60 caused transient hyperglycemia by itself and also aggravated the hyperglycemia induced by low-dose STZ. Thus, autoantibodies to insulin can indicate susceptibility to a toxic trigger of diabetes, and a low dose of a toxin can activate the insulin and hsp60 autoimmunity that has been detected previously in the spontaneous autoimmune diabetes of NOD strain mice.** *Diabetes* **43:992–998, 1994**

Autoimmune diabetes or insulin-dependent diabetes mellitus (IDDM), like many other autoimmune diseases of humans, requires a genetic predisposition, but its realization seems to be triggered by factors in the environment. Witness the low concordance of IDDM in monozygotic twins (1). An experimental model of autoimmune diabetes triggered by an external agent is that which is induced by a low dose of the β-cell toxin streptozotocin (STZ). A single high dose of STZ (200 mg/kg) directly poisons β-cells and causes an outbreak of clinical diabetes within 2–4 days. However, it is possible to induce diabetes with an autoimmune component by administration of five daily doses of 40 mg/kg each in genetically susceptible mice (2,3). Susceptible mice, such as males of the C57BL/KsJ (BKs) strain, develop diabetes ~3 weeks after such administration. Female mice of this strain are relatively resistant to the low-dose STZ-induced diabetes, as are both males and females of some other strains. Thus, administration of a suitable dose of a toxin to genetically susceptible subjects can lead to an autoimmune process.

An important question is the immunological relationship between the autoimmune process triggered by STZ and that characteristic of diabetes developing spontaneously. We have previously described immunity to insulin and to hsp60 (heat shock protein) during the period of insulitis that precedes overt diabetes in mice of the NOD strain (4). Moreover, we found that T-cells specific for the p277 peptide of hsp60 were a cause of diabetes in those mice (5). In our present study, we investigated immunity to insulin and to hsp60 in mice susceptible or resistant to autoimmune diabetes induced by low-dose STZ. To separate the autoimmune component from the toxic damage caused by STZ, we used a lower than usual dose of STZ and thereby prolonged the lag period between the toxic insult and the overt autoimmune diabetes. The results demonstrate immunological similarity between the autoimmune diabetes triggered by the toxin in male C57BL/KsJ mice and that developing spontaneously in NOD mice. These results are compatible with the idea that a standard module or blueprint of diabetogenic autoimmunity within the immune system can be triggered by different environmental agents (6).

### RESEARCH DESIGN AND METHODS

C57BL/KsJ (BKs) and BALB/c mice of both sexes were purchased from the Animal Resources Unit of the Jackson Laboratory (Bar Harbor, ME). The hybrids between BALB/c and BKs, (CXBKs)F1, were bred at the animal facilities of the Weizmann Institute of Science. All mice were housed under specific pathogen-free conditions and allowed free access to food (Purina Chow) and chlorinated drinking water.

**STZ-induced diabetes**. STZ (Boehringer Mannheim, Mannheim, Germany) was dissolved in 25 mM sodium citrate, pH 4.2, just before use and injected intraperitoneally in several dose regimes: 40 or 30 mg/kg of body weight for 5 consecutive days; a primary dose of 100 mg/kg followed by a dose of 40 mg/kg 2 weeks later; or 10 weekly doses of 10 mg/kg. At the times indicated, blood was obtained from the retroorbital

From the Department of Cell Biology (D.E., H.P., N.P., M.R., A.W.L., I.R.C.), the Weizmann Institute of Science, Rehovot; Meir Hospital (H.P.), Kfar-Saba; and Assaf-Harofe Hospital (M.R.), Nes-Ziona, Israel; and I. Medizinische Klinik (A.W.L.), Johannes Gutenberg Universitat, Mainz, Germany.

I.R.C. has been awarded a grant by Portman Pharmaceuticals Inc. to study therapy of IDDM.

Address correspondence and reprint requests to Dr. Irun R. Cohen, Department of Cell Biology, the Weizmann Institute of Science, Rehovot 76100, Israel.

Received for publication 5 January 1994 and accepted in revised form 14 April 1994.

STZ, streptozotocin; DM, Dana and Micha; hsp, heat shock protein; BSA, bovine serum albumin; SI, stimulation index; IDDM, insulin-dependent diabetes mellitus; IAA, insulin autoantibody; MHC, major histocompatibility complex.

sinus, and plasma glucose was determined using a Beckman Glucose Analyzer (Palo Alto, CA). Hyperglycemia was defined as a plasma glucose level >11.1 mM measured at 10:00 A.M. under non-fasting conditions. This concentration of glucose was chosen as the indicator of hyperglycemia because it was greater than three standard deviations above the mean plasma glucose concentration measured in 200 untreated mice (data not shown).

**Antigens**. Bovine insulin, bovine thyroglobulin, and bovine serum albumin (BSA) were obtained from Sigma (St. Louis, MO). Acetylation of insulin to acetyl$_3$ insulin was conducted using acetic anhydrid as described previously (7). Dr. Ruurd van der Zee (University of Utrecht, The Netherlands) supplied recombinant *M. tuberculosis* hsp60, human hsp60, and the pEX2 control for the recombinant antigens, a preparation of *E. coli* transfected with the plasmid not containing the hsp60 gene (8,9). The synthetic peptides p277 and p278 (5) were synthesized by Dr. Ora Goldberg of the biological services of the Weizmann Institute.

**Immunization to hsp60**. BKs male mice were injected subcutaneously with 50 μg of hsp60 emulsified in 0.2 ml of incomplete Freund's adjuvant (Difco, Detroit, MI) as previously described, and control mice were immunized with BSA administered in a similar fashion (4). In some experiments, groups of mice were given STZ 2 weeks later.

**Antibody measurement**. Naive or STZ-induced diabetic mice were bled at various times, and the sera were diluted 1:500 in 0.1% hemoglobin in phosphate-buffered saline and tested in a solid-phase radioimmunoassay as described previously (10). Briefly, microtiter PVC plates (Falcon, Becton Dickinson, Lincoln Park, NJ) were coated with hsp60 or insulin at 5 μg/ml for 18 h. Guinea pig anti-insulin antiserum from BioMakor (Rehovot, Israel), at a 1:100 dilution, was used as an antigen for the detection of anti-idiotypic antibodies (10). Nonspecific binding was blocked by a 2-h incubation with 1% hemoglobin, and the diluted serum samples were applied in triplicate for 2 h at 37°C followed by the second antibody ($^{125}$I-labeled goat anti-mouse immunoglobulin; Amersham, Amersham, U.K.) for 18 h at 40°C. The specifically bound radioactivity was counted in a γ-counter (Cobra, HP, Meriden, CT). The analysis of DM positive and DM negative insulin antibodies and the corresponding anti-idiotypic antibodies was performed in a similar manner using acetyl$_3$ insulin as an antigen for detection of DM negative insulin antibodies and native insulin for the detection of DM positive insulin antibodies as described previously (10). The sera were diluted 1:100 for these assays.

**T-cell responses**. T-cell proliferation was assayed using spleens obtained from STZ-induced diabetic mice as described previously (4,5). Splenocytes were seeded in quadruplicate wells in microtiter plates, 0.2 × $10^6$ in 0.2 ml of Dulbecco's modified Eagles's medium supplemented with 1% autologous serum for 72 h. Antigens were added at a concentration of 5 μg/ml. Wells were pulsed with [$^3$H]thymidine for the last 18 h, and the incorporated [$^3$H]thymidine was counted in a β-counter after the cells were harvested. The stimulation index (SI) was defined as the ratio of the antigen-driven incorporation of thymidine to the background incorporation of thymidine in the absence of antigen. Standard deviations were always <10% of the mean cpm.

Limiting dilution analysis of the numbers of reactive T-cells was conducted by preparing the splenocytes as above and plating them at concentrations of 10–8,000 cells per well (11). Irradiated (3,000 rad) syngeneic splenocytes from naive mice were added at 0.2 × $10^6$ per well as feeder cells. Each cell concentration was plated in 48 replicates, of which six served as background counts containing only cells and medium. Another six wells received 2.5 μg/ml concanavalin A to ascertain the maximum proliferative function of the tested splenocytes. Hsp60 (5 μg/ml) was added to the other 36 wells. Cells were incubated for 6 days, and [$^3$H]thymidine was added for the last 18 h. The cells were then harvested, the incorporated thymidine counted, and the percentage of positive wells (SI > 2) calculated for each point. The cell number corresponding to 37% negative wells was considered to represent the frequency of the T-cells responding to the antigen (12).

## RESULTS

**Spontaneous insulin autoantibodies associated with susceptibility to STZ: effect of strain and gender**. Autoantibodies to insulin are associated with the development of insulin-dependent diabetes mellitus (IDDM) in humans (13). Although autoantibodies to insulin are also found in the healthy population, they are more prevalent among first-degree relatives of IDDM patients (14). Thus, insulin autoantibodies (IAAs) appear to be a marker of susceptibility to IDDM. We therefore studied the spontaneous development of IAA with age as a possible correlation of the susceptibility of male and female BKs, BALB/c, and their F1 offspring to STZ-induced diabetes.

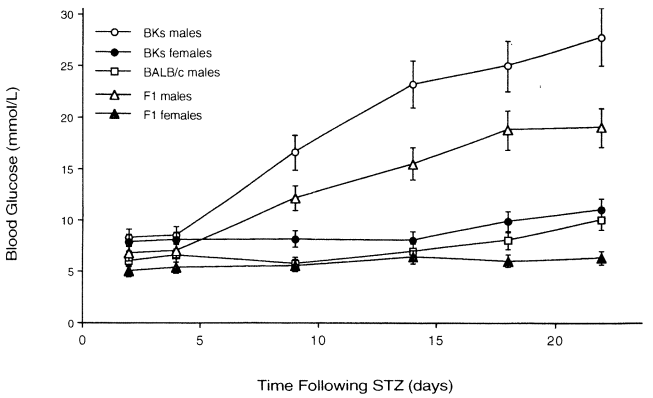


**FIG. 1. Strain and gender determine susceptibility to STZ-induced IDDM. Groups of 10 mice of various strains and sexes were given STZ 40 mg/kg daily for 5 days, and the blood glucose levels were tested in individual mice at the indicated times. Results are means ± SD of each group.**

Figure 1 shows the effect of multiple low-dose STZ (40 mg/kg daily for 5 days) on the blood glucose levels of BKs, BALB/c, and (CXBKs)F1 mice. Male BKs mice were highly susceptible to STZ-induced diabetes and male (CXBKs)F1 mice were moderately susceptible. BKs females, (CXBKs)F1 females, and BALB/c males and females were resistant to the induction of hyperglycemia by low-dose STZ. These results are compatible with the results of other studies (15).

The susceptible and resistant mice were then screened for IAAs and control antibodies in the absence of STZ administration (Fig. 2). We observed no differences between the mice in their antibodies to thyroglobulin (Fig. 2*B*), serum albumin, or kalikrein (data not shown). However, there was a marked difference in the spontaneous development of IAA (Fig. 2*A*). Male BKs and male (CXBKs)F1 mice showed a rise of IAA with age, beginning as early as 1 month. The IAA in the male (CXBKs)F1 mice seemed to level off, while the BKs IAA titer continued to rise with age. In contrast, the nonsusceptible mice did not show a spontaneous rise in titer of IAA. Thus, there seems to be an association of susceptibility to STZ-induced IDDM with a tendency to progressively develop IAA with age. Nevertheless, male BKs mice do not develop IDDM spontaneously even at 1 year of age (data not shown). Contact with the STZ toxin is required to trigger the transition from asymptomatic autoimmunity to clinical disease.

**Extending the autoimmune prodrome of IDDM**. The standard course of five divided doses of STZ, 40 mg/kg each, produces overt diabetes within 2–3 weeks. In contrast, the autoimmune prodrome is much longer in NOD mice. Clinical IDDM becomes manifest only 2–4 months after the onset of autoimmune insulitis (16). The shorter prodrome following low-dose STZ may result from a combination of autoimmunity and toxic damage caused by the STZ itself. Indeed, 1 day after the last of five STZ injections, a 64% decrease in islet volume was reported, indicating toxic inflammation of the islets (17). However, it has been demonstrated that reduced doses of STZ may prolong the lag time between administration of the toxin and the development of IDDM (18). Therefore, to separate the autoimmune process from the initiating

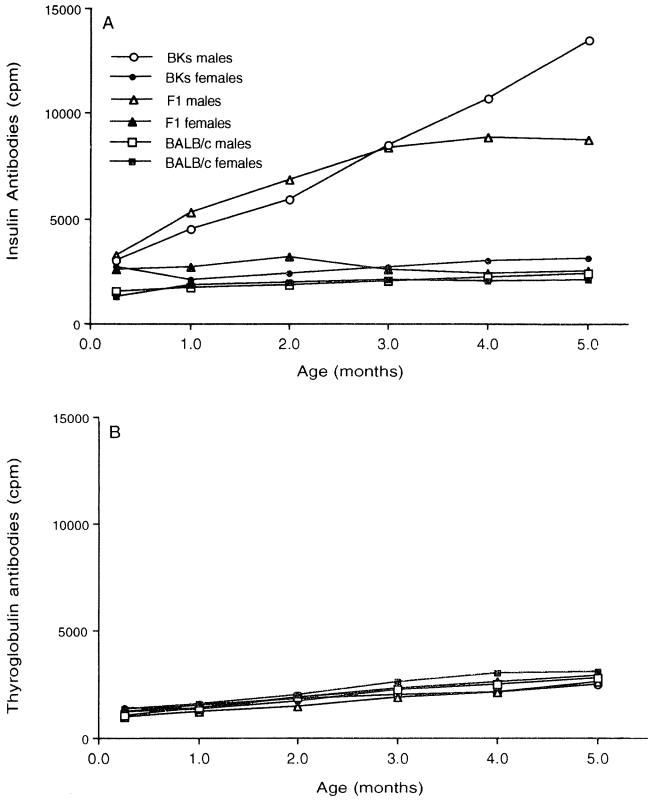


**FIG. 2. Spontaneous anti-insulin and anti-thyroglobulin antibodies. Groups of 10 mice of various strains were bled longitudinally and individually at the indicated ages, and the sera were tested for antibody titers to insulin (*A*) or to thyroglobulin (*B*). Results are mean cpm; SDs were <10% of the mean.**

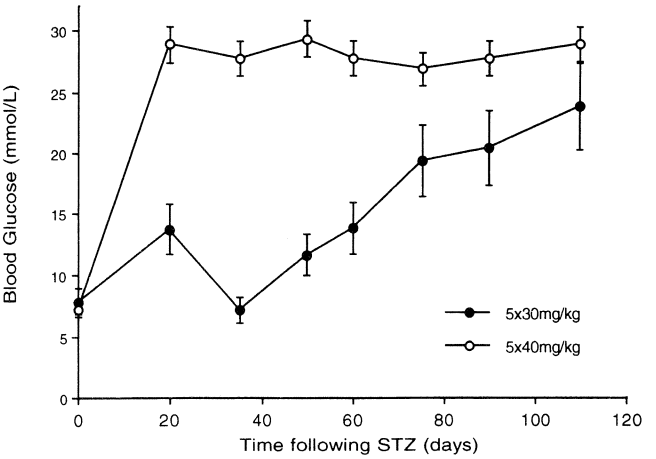


**FIG. 3. Dose effect of STZ-induced diabetes: reducing the dose of STZ to 30 mg/kg × 5 extends the lag phase of IDDM. Groups of 20 male BKs mice were given five daily doses of either 40 or 30 mg/kg. The concentration of blood glucose was measured as the mean ± SD at the indicated times.**

toxic insult, we investigated the induction of IDDM in BKs males using various doses and schedules of STZ. The following combinations were examined: *1*) 40 mg/kg for 5 consecutive days; *2*) 30 mg/kg for 5 consecutive days; *3*) 100 mg/kg once followed by a second dose of 40 mg/kg 2 weeks later; and *4*) 10 mg/kg once a week for 10 consecutive weeks.

Schedule 1, the standard treatment, produced hyperglycemia within 2–3 weeks as expected. Schedules 2 and 3 also produced hyperglycemia, but with a marked postponement of disease onset. Schedule 4 induced a mild persistent hyperglycemia of 11.1–13.9 mM that did not progress to severe IDDM.

Figure 3 shows a comparison between the standard 40 mg/kg × 5 dose and the 30 mg/kg × 5 dose. On day 20, the standard 40 mg/kg × 5 dose produced a maximal blood glucose level of 27.8 mM. In contrast, the 30 mg/kg × 5 dose induced only mild hyperglycemia (11–16.7 mM) by day 20. The blood glucose concentration returned to normal values and then rose again after 2 months to reach levels >22.2 mM by 3 months. Thus, the dose schedule of 30 mg/kg × 5 led to the development of IDDM long after administration of the STZ toxin. We chose to use the 30 mg/kg × 5 schedule because the extended prodrome allowed more time to document immunological changes at the preclinical stage of the disease.

**Autoantibodies after STZ**. Figure 4 shows the effects of STZ (30 mg/kg × 5) on the concentrations in male BKs mice of antibodies to thyroglobulin, to hsp60, and to insulin. Figure 4 also shows concentrations of anti-idiotypic antibodies to insulin antibodies of the DM positive idiotype. Idiotypic antibodies of the DM idiotype are detected by their binding to the conserved site of the insulin molecule that binds to the insulin receptor. Anti-DM idiotype antibodies therefore mimic the binding site of insulin and function as antibodies to the insulin receptor (10,19). Spontaneous anti-DM idiotype antibodies have been detected in the development of autoimmune IDDM in BB rats (20) and NOD mice (4), and in some newly diagnosed IDDM patients (21,22).

In this experiment, administration of STZ to male BKs mice led to two peaks of hyperglycemia: mild and remitting hyperglycemia between days 20 and 40 followed by severe IDDM only after 90 days. Coincident with the first wave of hyperglycemia at 20 days, rising titers of antibodies appeared to hsp60 and to insulin. Anti-idiotypic antibodies to the anti-DM antibodies rose later and peaked around day 70. There was no rise in thyroglobulin autoantibodies. Administration of STZ (30 mg/kg × 5) to female BKs mice failed to induce IDDM and also did not induce a rise in titers of antibodies to insulin or to hsp60 (data not shown).

Table 1 shows the relative concentrations of DM positive and DM negative anti-insulin antibodies and the anti-DM anti-idiotypic antibodies induced by STZ. Most of the pre-STZ natural anti-insulin antibodies were of the DM positive idiotype, and that contact with STZ first triggered an increase in these DM idiotype insulin antibodies followed by a rise in the anti-DM anti-idiotype antibodies. Thus, the diabetogenic process, which was triggered by STZ, was accompanied by specific autoantibodies to hsp60 and to insulin and by anti-idiotypic antibodies to insulin antibodies of the DM idiotype similar to the spontaneous autoimmune diabetes of the NOD strain (4).

**T-cell reactivity to hsp60**. We previously demonstrated that the spontaneous development of β-cell autoimmunity in NOD mice is accompanied by T-cells reactive to human hsp60 and to a defined peptide in the hsp60 molecule designated p277 (5). We therefore investigated the effect of low-dose STZ administration of male BKs mice on T-cell immunity to human hsp60.

Figure 5 shows that 25 days following STZ administration (30 mg/kg × 5), T-cell proliferative responses to hsp60 and to the p277 peptide of hsp60 could be demonstrated in male BKs mice (Fig. 5*A*). T-cell responses to hsp60 were detect-

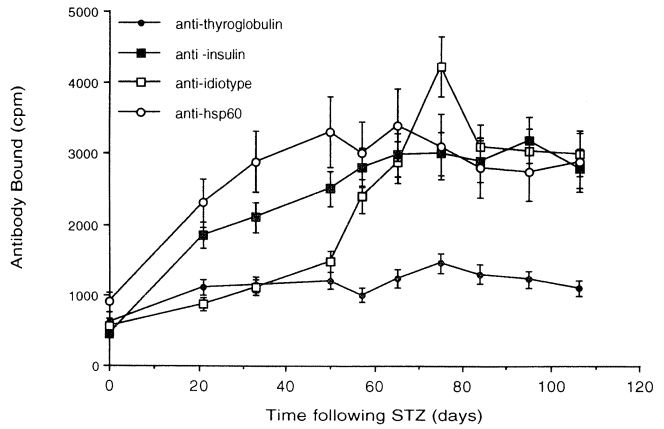


**FIG. 4. Sequential development of autoantibodies following STZ. Groups of 10 male BKs mice were given 30 mg/kg × 5 of STZ, and the titers of antibodies were measured in individual mice at the indicated times. Results are means ± SD.**

able throughout the period preceding overt diabetes, but their level did not rise markedly. Female BKs mice given STZ (30 mg/kg × 5) did not develop diabetes and did not manifest T-cell responses to hsp60 or to the p277 peptide (Fig. 5*B*). There also was no response to control peptide p278 (5), a peptide adjacent to p277 in the hsp60 sequence. Both male and female naive BKs mice had negligible T-cell responses to hsp60 and p277 (Fig. 5*A* and *B*). To quantitate the numbers of anti-hsp60 T-cells, we conducted a limiting dilution analysis of T-cell reactivity to human hsp60 in naive BKs and BALB/c mice and in BKs males after STZ administration. We observed a relatively high frequency of anti-hsp60 T-cells in naive mice: in BALB/c mice the frequency was 1:4,800, but in BKs males it reached 1:1,800. After STZ, the frequency rose to 1:200 at day 20 (Fig. 6). A lower frequency of 1:800 was found later, 40 days after STZ (data not shown). At both time points, the limiting dilution graph demonstrated a two-hit curve. Reactivity appeared to be lost when the cell number was reduced below 8,000 per well, but a second wave of positive wells emerged as the number of cells per well was further decreased. Two-hit kinetics in limiting dilution analysis have been attributed to the presence of regulatory cells (23). By day 80, the frequency of anti-hsp60 T-cells fell back to the levels seen in naive mice (data not shown).

TABLE 1
DM idiotypic and anti-idiotypic antibodies induced by STZ

| Time following STZ (5 × 30 mg/kg) (days) | Anti-insulin antibodies (cpm) DM positive idiotype | DM negative idiotype | Anti-DM anti-idiotypic antibodies (cpm) |
|---|---|---|---|
| 0 | 4,300 | 2,500 | 1,200 |
| 20 | 11,500 | 2,250 | 1,700 |
| 60 | 15,450 | 2,800 | 6,700 |
| 80 | 8,100 | 3,300 | 8,900 |
| 100 | 2,100 | 4,550 | 6,200 |

Data are mean cpm of the individual titers; SDs < 10% of mean. Twenty male BKs mice were induced to develop IDDM by administration of STZ (30 mg/kg × 5). The mice were bled individually on the indicated days, and the sera were tested for antibodies to insulin of the DM positive and DM negative idiotypes and for anti-idiotypic anti-DM positive antibodies. Day 0, the pre-bleed.

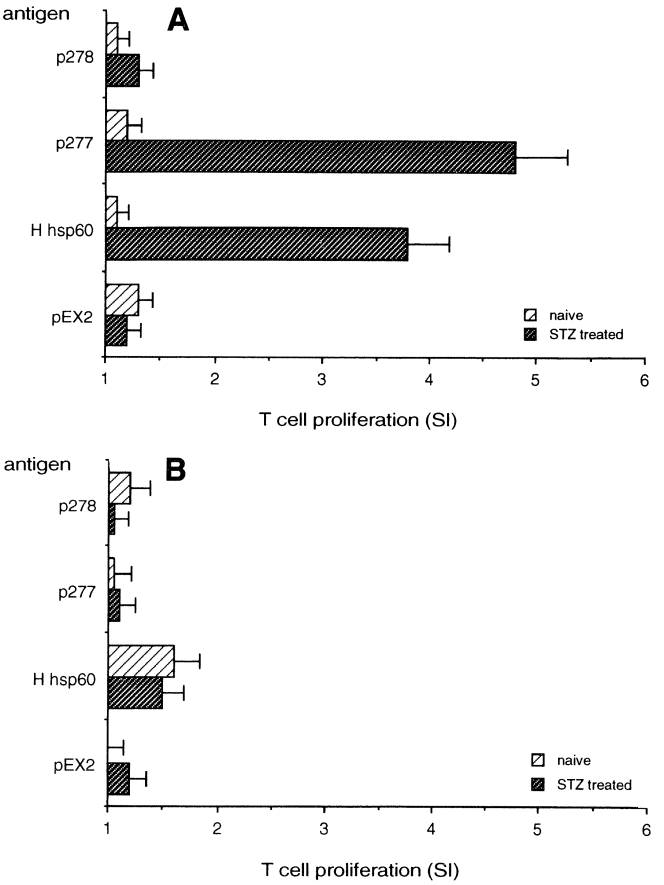


**FIG. 5. T-cell responses to hsp60 and p277 following STZ administration. Groups of 10 BKs male mice were given STZ (30 mg/kg × 5), and the proliferative responses of their individual spleens and of individual spleens from naive male BKs mice to the p277 and p278 peptides, to hsp60 (human), or to the pEX2 control were measured on day 25 (*A*). Female BKs mice were similarly induced (*B*).**

**Immunization to hsp60**. Active immunization of prediabetic NOD mice to hsp60 was found to cause an early onset of hyperglycemia and insulitis (4). Might immunization to hsp60 synergize with the STZ-induced diabetogenic process? Table 2 shows the blood glucose concentrations at various days of BKs mice immunized with human hsp60 14 days before STZ administration (30 mg/kg × 5). The combination of hsp60 immunization with STZ administration was marked by acceleration of the hyperglycemia observed following administration of the low-dose STZ alone. By 55 days after combined administration, the mice manifested blood glucose levels of ≥22 mM; the mice given STZ alone reached similar blood glucose levels only between 75 and 110 days. In other words, the toxin and the hsp60 immunogen synergised and aggravated the diabetogenic process. Immunization to BSA did not have an effect on the disease.

## DISCUSSION

The experiments described demonstrate that *1*) spontaneous development of IAA of the DM idiotype marks susceptibility to clinical IDDM induced by a low dose of the STZ toxin; *2*) genes and gender influence the disposition to insulin autoimmunity and to STZ-induced IDDM; *3*) the toxin-induced disease is associated with a rise in IAA of the DM idiotype and anti-DM anti-idiotypic antibodies and with anti-hsp60 antibodies and T-cells; *4*) STZ activates anti-hsp60 T-cells that recognize the p277 peptide, a target of diabeto-

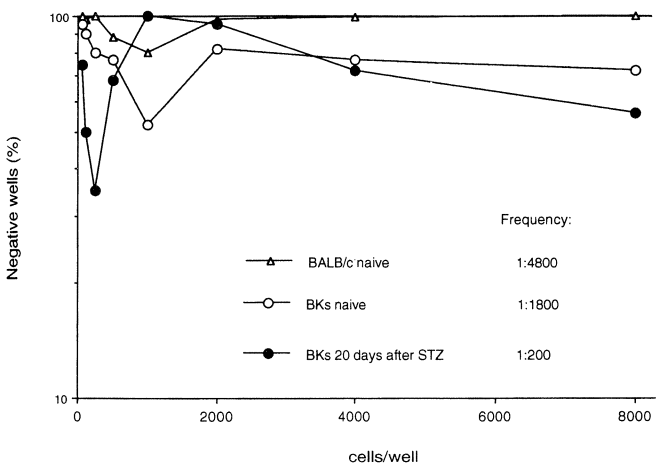


**FIG. 6. Limiting dilution analysis of T-cells responsive to hsp60. Groups of 10 BKs males were given STZ (30 mg/kg × 5), and their pooled spleen cells were tested by limiting dilution analysis on day 20 for reactivity to human hsp60. Naive male BKs and BALB/c mice of the same age were used as controls.**

genic T-cells in the spontaneous IDDM of the NOD mouse; and *5*) immunization to hsp60 can aggravate the IDDM triggered by the STZ toxin. Whether STZ also triggers immunity to other β-cell antigens thought to be involved in IDDM, such as glutamic acid decarboxylase (24,25) or the 38 kDa antigen (26), is unclear.

What is the origin of the IAAs that mark susceptibility to STZ-induced autoimmunity? Serreze et al. (27) described molecular mimicry between p73, an endogenous retroviral gene, and insulin. The BKs mice we used in our experiments, which do not carry the *db* mutation present in the mice studied by Serreze et al., nevertheless do express p73 in the thymus and in the β-cells (28); hence, the spontaneous IAA we found in our BKs mice might arise as a consequence of inherent p73 expression.

Whatever the cause of the autoantibodies, the association of STZ-induced diabetes with the spontaneous development of IAA suggests that deficiencies in immune regulation can combine with an environmental insult, such as STZ, to produce clinical IDDM. Administration of low-dose STZ to BALB/c or to female BKs mice, which do not express a high titer of IAA, does not induce immunological diabetes. Similarly, the IAAs by themselves in the susceptible mice do not make disease inevitable: they represent benign autoimmunity that requires the toxic insult to induce the transition to overt disease.

Both spontaneous IAA and susceptibility to multiple low-dose STZ-induced diabetes are related to gender and to genes outside of the major histocompatibility complex (MHC): resistant BALB/c and BKs female mice share the H-$2^d$ genotype with the susceptible BKs and (CxBKs)F1 males. Moreover, the association between the various H-2 haplotypes and STZ-induced IDDM varies with the non-MHC genetic background (29–31). The sexual dimorphism observed in both BKs and (CXBKs)F1 mice is a common phenomenon of the low-dose STZ model, probably related to differences in sex steroid metabolism as reviewed by Wilson and Leiter (32).

TABLE 2
Immunization to hsp60 accelerates STZ-induced diabetes

| Time after administration (Days) | Blood glucose (mM) | | |
|---|---|---|---|
| | STZ | BSA + STZ | hsp60 + STZ |
| 30 | 11.1 ± 2.3 | 9.9 ± 1.5 | 16.1 ± 1.7 |
| 42 | 11.4 ± 1.9 | 10.5 ± 2.1 | 18.1 ± 2.0 |
| 55 | 9.4 ± 1.5 | 10.8 ± 1.9 | 22.2 ± 2.9 |
| 75 | 19.7 ± 3.8 | 18.9 ± 2.7 | 26.7 ± 3.2 |
| 110 | 23.9 ± 4.2 | 25.2 ± 3.3 | 27.8 ± 3.9 |

Data are means ± SD. Groups of 10 male BKs mice were given STZ (30 mg/kg × 5) with or without having been immunized 10 days earlier either with human hsp60 or with BSA (50 μg in incomplete Freund's adjuvant given subcutaneously). Blood glucose levels were measured in the individual mice at the indicated times.

Anti-DM anti-idiotypic antibodies to the IAA, which appear spontaneously in NOD mice (4), in BB rats, and in some patients (20–22), can also be detected in nondiabetic mice actively immunized to insulin (33). These anti-idiotypic antibodies cause desensitization of the insulin receptor and might aggravate the diabetogenic process by adding peripheral insulin resistance to the central destruction of β-cells (34).

Importantly, the multiple low-dose STZ model demonstrates that anti-hsp60 immunity may be a factor in toxin-induced IDDM as well as in the spontaneous IDDM of NOD mice (4,5). The hsp60 molecule has been localized to the secretory granules of β-cells by Brudzynski et al. (35,36). Thus, anti-hsp60 immunity might preferentially affect the β-cell because of the unique expression of this antigen in the insulin secretory apparatus. Other tissues generally express hsp60 in the mitochondria (37,38) or in the cytoplasm following stress (39). It is conceivable that STZ, as a β-cell-specific toxin, amplifies β-cell expression of hsp60 and makes these cells more susceptible to hsp60-specific autoimmunity. We reported that anti-hsp60 T-cell clones can adoptively transfer hyperglycemia and insulitis into prediabetic NOD mice and into nondiabetic NON.H-$2^{nod}$ mice (5). As shown here, the development of IDDM induced by low-dose STZ is accompanied by the activation of anti-hsp60 T-cells (Fig. 5). T-cell reactivity to the p277 peptide of hsp60, which is critical in the diabetes of NOD mice (5,40), was also detected in the present STZ model. Active immunization to hsp60 was found to synergize with STZ to accelerate the appearance of IDDM (Table 2). Moreover, active immunization of BKs mice to hsp60 can induce a bout of hyperglycemia even in the absence of STZ administration (D.E., unpublished observations). Thus, hsp60 immunity is functional in the diabetogenic process.

There is evidence for the induction of a neoantigen by STZ; for example, transplanted islets need to be exposed to STZ for immunological rejection by STZ-induced mice (41). Moreover, T-cells can distinguish between control and STZ-induced cells (42). However, to date, only viral antigens have been reported to be induced by STZ administration (43). A putative target antigen induced by STZ that is related to hsp60 is conceivable.

The limiting dilution analysis of the number of anti-hsp60 T-cells revealed a high frequency of such cells (1:200) in the periphery (Fig. 6). This frequency is similar to that of T-cells responsive to myelin basic protein in experimental autoimmune encephalomyelitis (11), a disease known to be caused by T-cells (44). However, the present limiting dilution analysis showed a two-hit curve. This suggests that regulatory cells might downregulate the anti-hsp60 T-cells. Anti-idiotypic regulatory cells have been reported to downregulate experimental autoimmune encephalomyelitis (45,46). It re-

mains to be seen whether the regulatory cells in IDDM are also anti-idiotypic.

Interestingly, the autoimmune diabetes triggered by low-dose STZ in BKs mice was immunologically similar to the autoimmune diabetes developing in NOD mice. Both include, in the prodrome period, autoantibodies to insulin of the DM idiotype, anti-DM anti-idiotypic antibodies, antibodies and T-cells to hsp60, and T-cells to the p277 peptide of hsp60. The spontaneous autoimmune diabetes of BB rats also manifests anti-insulin antibodies of the DM idiotype and anti-idiotypic anti-DM antibodies (20). Anti-idiotypic antibodies to insulin occur in human diabetes (21,22). We have also found anti-hsp60 immunity in humans with newly diagnosed IDDM (40 and D.E., unpublished observations). Thus, a similar set of immune reactivities may characterize autoimmune diabetes produced by different mechanisms in various species. The reasons for the immunological prominence of insulin and hsp60 are unknown, but the phenomenon is compatible with the notion that certain standard elements of autoreactivity are built into the immune system naturally, a notion embodied as the immunological homunculus (47). Dysregulation of this benign natural autoimmunity, by low-dose STZ in BKs mice, or by unknown environmental factors in NOD mice or in susceptible humans, causes its transition into progressive, autoimmune destruction of β-cells culminating in clinical diabetes.

## ACKNOWLEDGMENTS

Parts of this study were supported by the Minerva Foundation, the Juvenile Diabetes Foundation International, the Tauro Foundation, and by a gift from Rowland Schaeffer. D.E. was a postdoctoral fellow of the Juvenile Diabetes Foundation International, and I.R.C. is the incumbent of the Mauerberger Chair in Immunology and director of the Robert Koch-Minerva Center for Research in Autoimmune Disease.

## REFERENCES

1. Olmos P, A'Hern R, Heaton DA, Millward BA, Risley D, Pyke DA, Leslie RD: The significance of the concordance rate for type I (insulin-dependent) diabetes in identical twins. *Diabetologia* 31:747–750, 1988
2. Like AA, Rossini AA: Streptozotocin-induced pancreatic insulitis: new model of diabetes mellitus. *Science* 193:415–417, 1976
3. Rossini AA, Appel M, Williams RM, Like AA: Genetic influence of streptozotocin-induced insulitis and hyperglycemia. *Diabetes* 26:916–920, 1977
4. Elias D, Markovits D, Reshef T, van der Zee R, Cohen IR: Induction and therapy of autoimmune diabetes in the non-obese diabetic (NOD/Lt) mouse by a 65-kDa shock protein. *Proc Natl Acad Sci USA* 87:1576–1580, 1990
5. Elias D, Reshef T, Birk OS, van der Zee R, Walker MD, Cohen IR: Vaccination against mouse diabetes with a T-cell epitope of the human 65 kDa heat shock protein. *Proc Natl Acad Sci USA* 88:3088–3091, 1991
6. Eisenbarth GS: Type I diabetes mellitus: a chronic autoimmune disease. *N Engl J Med* 314:1360–1368, 1986
7. Shechter Y, Elias D, Bruck R, Maron R, Cohen IR: Mice immunized to insulin develop anti-idiotypic antibody to the insulin receptor. In *Anti-Idiotypes, Receptors, and Molecular Mimicry.* DS Linthicum, NR Farid, Eds. New York, Springer-Verlag, 1988, p. 71–91
8. Thole JER, Keulen WJ, Kolk AHJ, Groothius DG, Berwald LG, Tiesjema RH, van Embden JDA: Characterization, sequence determination, and immunogenicity of a 64-kilodaton protein of *Mycobacterium bovis* BCG expressed in *Escherichia coli* K-12. *Infect Immunol* 55:1466–1475, 1987
9. van Eden W, Thole JER, van der Zee R, Noordzji A, van Embden JDA, Hensen EJ, Cohen IR: Cloning of the mycobacterial epitope recognized by T-lymphocytes in adjuvant arthritis. *Nature* 331:171–173, 1988
10. Cohen IR, Elias D, Rapoport M, Shechter Y: Anti-idiotypic antibodies and molecular mimicry: the production and properties of anti-idiotypic antibodies that recognize the insulin receptor. *Methods Enzymol* 178: 300–307, 1989
11. Mor F, Cohen IR: T-cells in the lesion of experimental autoimmune encephalomyelitis. *J Clin Invest* 90:2447–2455, 1992
12. Antoniou AV, Parker D, Turk JL, Tan BG, Scheper RJ: Immunocytochemical identification and quantification of mononuclear cells in the meninges during the development of chronic relapsing experimental allergic encephalomyelitis (CREAE) in the guinea pig. *Cell Immunol* 97:386–396, 1986
13. Palmer JP, Asplin CM, Clemons P, Lyon K, Tapati O, Raghu R, Paquette TL: Insulin antibodies in insulin-dependent diabetes before insulin treatment. *Science* 222:1337–1339, 1983
14. Nell LJ, Hulbert C, Arem R, Marshall RN, Rogers DG, Comstock JP, Ellerhorst JA, Thomas JW: Factors affecting the insulin autoantibody ELISA. *Autoimmunity* 2:299–309, 1989
15. Maclaren NK, Neufeld M, McLaughlin JV, Taylor G: Androgen sensitization of streptozotocin-induced diabetes in mice. *Diabetes* 29:710–716, 1980
16. Miyazaki A, Hanafusa T, Yamada K, Miyagawa J, Fujino-Kurihara H, Nakajima H, Nonaka K, Tarui S: Predominance of T-lymphocytes in pancreatic islets and spleen of pre-diabetic non-obese diabetic (NOD) mice: a longitudinal study. *Clin Exp Immunol* 60:622–630, 1985
17. Bonnevie-Nielsen V, Steffes MW, Lernmark A: A major loss in islet mass and B-cell function precedes hyperglycemia in mice given multiple low doses of streptozotocin. *Diabetes* 35:1027–1033, 1986
18. Riley JW, McConnell TJ, Maclaren NK, McLaughlin JV, Taylor G: The diabetogenic effects of streptozotocin in mice are prolonged and inversely related to age. *Diabetes* 30:718–723, 1981
19. Shechter Y, Elias D, Maron R, Cohen IR: Mouse antibodies to the insulin receptor developing spontaneously as anti-idiotypes. I. Characterization of the antibodies. *J Biol Chem* 259:6416–6419, 1984
20. Elias D, Bone AJ, Baird JD, Cooke A, Cohen IR: Insulin-mimicking anti-idiotypic antibodies in development of spontaneous autoimmune diabetes in BB/E rats. *Diabetes* 39:1467–1471, 1990
21. Maron R, Elias D, DeJong BM, Bruining GJ, van Rood J, Shechter Y, Cohen IR: Autoantibodies to the insulin receptor in juvenile onset insulin-dependent diabetes. *Nature* 303:817–818, 1983
22. Casiglia D, Giardina E, Triolo G: IgG auto-anti-idiotype antibodies against antibody to insulin in insulin-dependent (type 1) diabetes mellitus: detection by capture enzyme-linked immunosorbent assay (ELISA) and relationship with anti-insulin antibody level. *Diabetes Res* 16:181–184, 1991
23. Fey K, Melchers I, Eichmann K: Quantitative studies on T-cell diversity. IV. Mathematical analysis of multiple limiting populations of effector and suppressor T-cells. *J Exp Med* 158:40–52, 1983
24. Baekkeskov S, Aansroot HJ, Christgau S, Reetz A, Solimena M, Cascalho M, Folli F, Richter-Olesen H, De Camilli P: Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. *Nature* 347:151–156, 1990
25. Atkinson MA, Kaufman DL, Campbell L, Gibbs KA, Shan SC, Bu D-F, Erlander MG, Tobin AJ, Maclaren NK: Response of peripheral-blood mononuclear cells to glutamate decarboxylase in insulin-dependent diabetes. *Lancet* 339:458–459, 1992
26. Roep BO, Arden SD, de Vries RRP, Hutton JC: T-cell clones fron type 1 diabetes patient respond to insulin secretory granule proteins. *Nature* 345:832–834, 1990
27. Serreze DV, Leiter EH, Kuff EL, Jardieu P, Ishizaka K: Molecular mimicry between insulin and retroviral antigen. *Diabetes* 37:351–358, 1988
28. Kuff EL, Fewell JW: Intracisternal A particle gene expression in normal mouse thymus tissue: gene products and strain related variability. *Mol Cell Biol* 5:474–483, 1985
29. Kiesel U, Falkenberg FW, Kolb H: Genetic control of low-dose streptozotocin-induced autoimmune diabetes in mice. *J Immunol* 130:1719–1722, 1983
30. Wolf J, Lily F, Shin S-I: The influence of genetic background on the susceptibility of inbred mice to streptozotocin-induced diabetes. *Diabetes* 33:567–571, 1984
31. Tanaka S-I, Nakajima S, Inoue S, Takamura Y, Aoki I, Okuda K: Genetic control by I-A subregion in H-2 complex of incidence of streptozocin-induced autoimmune diabetes in mice. *Diabetes* 39:1298–1304, 1990
32. Wilson GL, Leiter EH: Streptozotocin interactions with pancreatic β-cells and the induction of insulin-dependent diabetes. *Curr Top Microbiol Immunol* 156:27–54, 1990
33. Shechter Y, Maron R, Elias D, Cohen IR: Autoantibodies to the insulin receptor spontaneously develop as anti-idiotypes in mice immunized with insulin. *Science* 216:542–545, 1982
34. Elias D, Maron R, Cohen IR, Shechter Y: Mouse antibodies to the insulin receptor developing spontaneously as anti-idiotypes. II. Effect on glucose homeostasis and the insulin receptor. *J Biol Chem* 259:6411–6415, 1984
35. Brudzynski K, Martinez V, Gupta RS: Secretory granule autoantigen in insulin-dependent diabetes mellitus is related to 62 kDa heat-shock protein (hsp60). *J Autoimmun* 5:453–463, 1992
36. Brudzynski K, Martinez V, Gupta RS: Immunocytochemical localization of heat shock protein 60–related protein in β-cell secretory granules and its altered distribution in non-obese diabetic mice. *Diabetologia* 35:316–324, 1992
37. Reading SD, Hallberg LR, Myers AM: Characterization of the yeast HSP60 gene coding for mitochondrial assembly factor. *Nature* 337:655–659, 1989
38. Ostermann J, Horwich AL, Neupert W, Hartl FU: Protein folding in the mitochondria requires complex formation with hsp60 and ATP hydrolysis. *Nature* 341:125–130, 1989
39. Polla BS: A role for heat shock proteins in inflammation? *Immunol*

*Today* 9:134–137, 1988
40. Elias D, Cohen IR: Peptide therapy for diabetes in NOD mice. *Lancet* 343:704–706, 1994
41. Weide LG, Lacy PE: Low-dose streptozocin-induced autoimmune diabetes in islet transplantation model. *Diabetes* 40:1157–1162, 1991
42. Klinkhammer C, Popowa P, Gleichmann H: Specific immunity to streptozocin. *Diabetes* 37:74–80, 1988
43. Appel MC, Rossini AA, Williams RM, Like AA: Viral studies in streptozotocin-induced pancreatic insulitis. *Diabetologia* 15:107–114, 1978
44. Ben-Nun A, Wekerle H, Cohen IR: The rapid isolation of clonable antigen-specific T-lymphocyte lines capable of mediating autoimmune encephalomyelitis. *Eur J Immunol* 11:195–199, 1981
45. Lider O, Reshef T, Beraud E, Ben-Nun A, Cohen IR: Anti-idiotypic network induced by T-cell vaccination against experimental autoimmune encephalomyelitis. *Science* 239:843–845, 1988
46. Sun D, Quin Y, Chluba J, Epplen JT, Wekerle H: Suppression of experimentally induced autoimmune encephalomyelitis by cytolytic T-cell interactions. *Nature* 332:843–845, 1988
47. Cohen IR: The cognitive paradigm and the immunological homunculus. *Immunol Today* 13:490–494, 1992