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Anti-pancreatic immunity in genetically diabetic mice

MONIQUE DEBRAY-SACHS, P. SAÏ, C. BOITARD, R. ASSAN & J. HAMBURGER *Institut National de la Santé et de la Recherche Médicale, Unité 25 and Laboratoire associé du Centre National de la Recherche Scientifique, Hôpital Necker, Paris and Service de Diabétologie, Hôpital Bichat, Paris, France*

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SUMMARY

The anti-pancreatic immune reaction of genetically diabetic homozygote C57Bl/KsJ db/db mice was studied with an *in vitro* test using murine islet of Langerhans cells as target cells. C57Bl/KsJ db/db spleen lymphocytes inhibited insulin secretion by the islet cells. This inhibition was abolished when T cells were eliminated by treatment with anti-Thy 1.2 monoclonal antibody in the presence of complement. Together with this cell-mediated cytotoxicity, complement-dependent antibody (CDA) and antibody-dependent cell cytotoxicity (ADCC) were found in the sera of these mice. A longitudinal study showed that this anti-pancreatic toxicity was detectable as early as the 10th day of life and lasted throughout the entire life span of the animal. None of these anomalies was found in control heterozygote mice.

INTRODUCTION

Various models of spontaneous diabetes mellitus have been documented in laboratory rodents (review in Mordes & Rossini, 1981). Recent works have suggested that autoimmune phenomena may be present in some of these animals, particularly the BB Worcester rat (Like *et al.*, 1979). In the C57Bl/KsJ mouse strain, the autosomal recessive mutation db produces severe diabetes mellitus, associated with hyperinsulinaemia during the earliest stage of the syndrome (Coleman & Hummel, 1967; Staats, 1975; Coleman, 1978). A β cell necrosis occurs later, associated with insulinopaenia and more pronounced hyperglycaemia (Mordes & Rossini, 1981).

In earlier studies in the mouse we verified that β cells from isolated islet cells could be stimulated *in vitro* with glucose and theophylline and that the subsequent insulin response was suppressed by specifically immunized mononuclear cells or activated macrophages (Debray-Sachs *et al.*, 1978, 1981). Similar findings were reported in the human with mononuclear cells (Boitard *et al.*, 1981a, 1981b) and sera (Saï *et al.*, 1981) from insulin-dependent diabetics.

The aim of the present study was to make a longitudinal analysis of autoimmune anti-pancreatic mechanisms during the life span of C57Bl/KsJ db/db mice. Controls were heterozygote C57Bl/KsJ db/+ mice.

MATERIALS AND METHODS

Animals. The experimental animal was C57Bl/KsJ db/db (homozygote) (H-2^d). Controls were heterozygotes CS7Bl/KJ db/+. Animals used as a source of pancreatic target cells were DBA/2

Correspondence: Monique Debray-Sachs, Centre de Recherches Néphrologiques de l'Hôpital Necker, 161 Rue de Sèvres, 75730 Paris Cedex 15, France.

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(H-2^d), C57Bl/6 (H-2^b), C3H (H-2^k) mice and Lewis rats. (Centre de Sélection d'Elevage des Animaux de Laboratoire du C.N.R.S., Orléans la Source, France).

Evaluation of the diabetic state. Weight, blood glucose and plasma insulin were screened at 9 am in 12 hr fasted mice.

Insulin/glucose index. Plasma insulin was plotted versus the concomitant blood glucose in order to calculate an insulin/glucose index = plasma insulin μU/ml/blood glucose mmol/l.

Preparation of spleen lymphocytes. Animals were anaesthetized with ether and killed by exsanguination. Spleens were removed and cell suspensions were prepared by disruption with forceps. After washings with Hank's balanced salt solution (HBSS), spleen cells were counted and adjusted to a final concentration of 4×10^6 viable cells/ml in minimum essential medium (MEM) with Earle's salts (Flow Laboratories, Inc., Rockville, Maryland, USA) supplemented with 1% sodium puruvate 100 × (Flow Laboratories, Inc.), 1% L-glutamine (Flow Laboratories, Inc.), 100 iu/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum.

Monoclonal anti-mouse Thy 1.2 antibody. Monoclonal anti-mouse Thy 1.2 antibody was obtained from NEN (New England Nuclear, Boston, Massachusetts, USA). Cell suspensions (9×10^6 cells/300 μl HBSS) were incubated for 15 min at 37°C with 30 μl of pure monoclonal antibody. Mouse absorbed rabbit serum (27 μl) was then added. After 1 hr of incubation at 37°C cells were washed twice with HBSS. Control treatments were performed by replacing monoclonal antibody with 30 μl of HBSS. The trypan blue exclusion test, performed after monoclonal antibody and complement treatments, showed an increased cell mortality from 25 to 35% when compared with control samples.

Preparation of pancreatic islet cells. The method used was previously described by Boitard *et al.* (1981a, 1981b). Briefly, pancreatic islets were isolated from DBA/2 mice as described by Lacy & Kostianovsky (1967). In some experiments, C57Bl/6 (H-2^b) or C3H (H-2^k) mouse and Lewis rat islets were also used. Cell suspensions were prepared according to the method of Ono, Takaki & Fukuma (1977). The cells were suspended in culture medium as described above, at a concentration of 5×10^4 cells/ml and cultured in flat bottomed microplates (Microtest II, Falcon Labware, Div. of Becton-Dikinson & Co.) with 5×10^3 cells/well in 0·1 ml plus lymphocytes (4×10^5 well in 0·1 ml) or medium (0·1 ml). The microplate was incubated for 18 hr at 37°C in 5% CO_2 and humidified air.

Glucose stimulation. At the end of the incubation period, the wells were washed and the supernatants were replaced by basal or stimulatory medium. The stimulatory medium consisted of MEM supplemented with 16·5 mmol/l glucose and 5 mmol/l theophylline (Sigma, St Louis, Missouri, USA); plates were kept at 37°C during the stimulation. Five minutes after adding the basal or stimulatory medium, supernatants were collected and rapidly frozen for insulin determinations. All experiments were performed in sextuplicate.

Antibody-dependent cell cytotoxicity (ADCC). Following distribution of the islet cell suspension into Falcon microtest II plates, 10 μl of control or diabetic heat-inactivated sera diluted 1/50 were added. After 45 min, effector cells, normal human lymphocytes obtained by separation on a Ficoll-Hypaque density gradient, were added (4×10^5/well in 0·1 ml) and the plate tray was placed in an incubator for 18 hr at 37°C in 5% CO_2 and humidified air. At the end of the incubation, glucose stimulation was carried out as described above.

Secretion index. In order to compare the insulin release from different islet cell preparations, a secretion index was calculated:

$$\text{Secretion index} = \frac{\text{stimulated release} - \text{basal release}}{\text{basal release}} \times 100.$$

Complement-dependent antibodies (CDA). The technique was previously described by Saï *et al.* (1981) and used entire islets instead of cells as targets. Islets were stimulated for 30 min, using the same stimulatory medium as above.

Insulin concentration. Insulin concentration was determined by radioimmunoassay as described by Yalow & Berson (1960).

RESULTS

The weight of diabetic mice was not statistically different from that of controls at 10 days of age. It

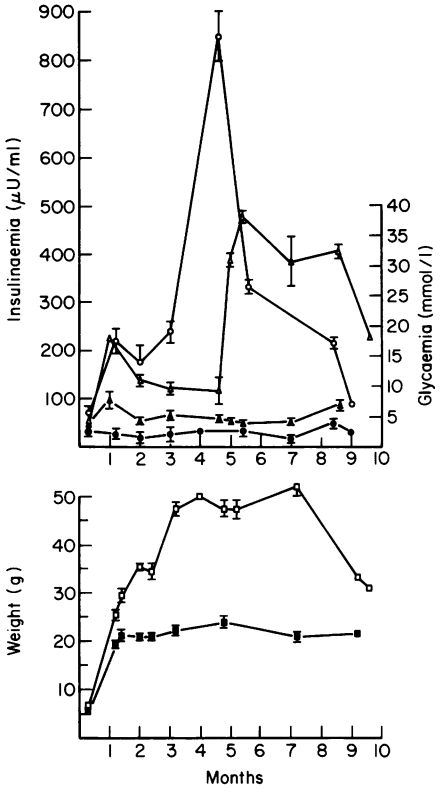

Fig. 1. Glycaemia (○, ●), insulinaemia (△, ▲) and weight (□, ■) during the life of C57Bl/KsJ db/db (○, △, □) and C57Bl/KsJ db/db (●, ▲, ■) mice respectively.

was higher than that of their non-diabetic counterparts from the 5th week to the 9th month at the end of study. On day 10, blood glucose levels were normal. At all subsequent stages (from 5 weeks onward) blood glucose was significantly higher in the C57Bl/KsJ db/db mice than in controls; hyperglycaemia was more pronounced after the 5th month of age than during earlier periods. Plasma insulin was significantly higher in the diabetic mice than in controls. Hyperinsulinaemia was found as early as the 10th day of life: it was more pronounced before the 5th month than during later stages (Fig. 1).

The insulin/glucose index was higher in diabetic mice than in controls. It decreases with time but, in our series, remained higher than normal throughout our study (up to 9 months of age) (Table 1).

Table 1. Insulin/glucose index

	Insulin/glucose index*								
	10 days	5/6 weeks	2 months	3 months	4 months	5 months	7 months	8 months	9 months
Control mice	4·5	4·5	4·5	5	9	8	5·5	6	5
Diabetic mice	13	15	27·5	41	75	35	4·5	5	4·5

*This was calculated as described in Materials and Methods.

Insulin release in the presence of spleen lymphocytes

Influence of total spleen cells. Incubation with control spleen cells from heterozygote mice did not modify the β cell response to stimulatory medium, whatever the age of lymphocyte donor mice.

Following incubation with diabetic spleen cells, the β cell response to glucose + theophylline was suppressed. The inhibition of β cell responses by diabetic spleen cells was found as early as the 10th day of age and consistently thereafter (Table 2).

Effect of monoclonal anti-mouse Thy 1.2 antibody treatment. When spleen cells were pre-treated with monoclonal anti-mouse Thy 1.2 antibody plus complement, they no longer inhibited insulin release (Fig. 2). The same treatment applied to control spleen cells did not modify the β cell response.

Influence of the major histocompatibility complex H-2. The DBA/2 mice used for islet preparations share the same H-2 locus (H-2^d) with the C57Bl/KsJ strain. However, identical results were obtained with islet cells prepared from C57Bl/6 (H-2^b), C3H (H-2^k) mice and Lewis rats (Table 3).

Insulin release in the presence of complement and normal or diabetic sera (CDA)

Again expressed as a stimulation index, the mean secretion index was 333% when islets were incubated with control sera ($n=5$; $P<0{\cdot}001$). After incubation with diabetic mouse sera the mean secretion index was 25% ($n=5$; ns). When complement was omitted the mean secretion index was 300% ($n=5$; $P<0{\cdot}001$).

Table 2. Insulin secretion index

	Insulin secretion index* (%)					
Age of mice	10 days	4/6 weeks	3/4 months	5 months	6 months	7 months
Islet cells + control lymphocytes	102 ($n=3$)	92 ($n=17$)	123 ($n=8$)	75 ($n=5$)	190 ($n=3$)	79 ($n=3$)
Islet cells + diabetic lymphocytes	0 ($n=3$)	0 ($n=10$)	0 ($n=8$)	0 ($n=5$)	0 ($n=3$)	0 ($n=3$)

*This was calculated as described in Materials and Methods.

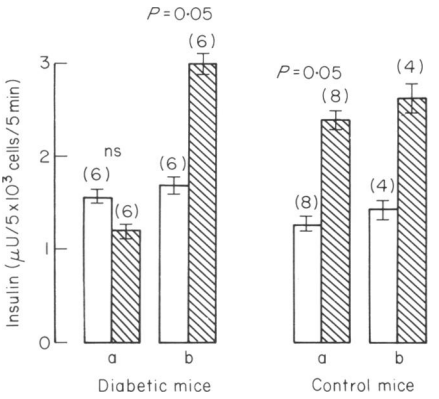

Fig. 2. β cell responses to glucose + theophylline in the presence of total spleen cells (a) and spleen cells pretreated with monoclonal anti-mouse Thy 1.2 antibody plus complement (b). Results are presented as mean values ± s.e.m. Number of experiments appears in parentheses. White columns represent the basal insulin release and hatched columns represent the stimulated release.

Table 3. Role of the major histocompatibility complex on the insulin suppressive potency of spleen cells

Origin of spleen cells	Origin of islet cells* (%) DBA/2 mouse (H-2^d)	C57Bl/6 mouse (H-2^b)	C3H mouse (H-2^k)	Lewis rat (Ag B1)
C57Bl/KsJ db/db mouse	0	0	0	9
C57Bl/KsJ db/+ mouse	163	86	145	100
C57Bl/6 mouse	45	112	159	122
DBA/2 mouse	90	160	205	184

*A typical experiment. All results are expressed in insulin secretion index.

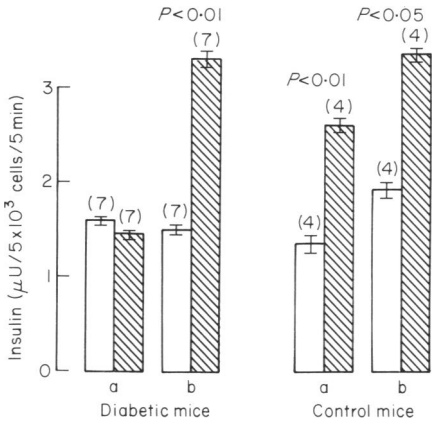

Fig. 3. β cell responses to glucose + theophylline in the presence of diluted serum + human lymphocytes (a) or diluted serum alone (b). Results are presented as mean values ± s.e.m. Number of experiments appears in parentheses. White columns represent the basal insulin release and hatched columns the stimulated release.

Insulin release in the presence of human mononuclear cells plus normal or diabetic sera (ADCC)
Diabetic sera, heat-inactivated at 56°C for 30 min and diluted 1/50, were not cytotoxic to islet cells. The basal insulin release was $1{\cdot}74 \pm 0{\cdot}23$ $\mu U/5 \times 10^3$ cells/5 min and the stimulated release was significantly higher: $3{\cdot}30 \pm 0{\cdot}46$ $\mu U/5 \times 10^3$ cells/5 min ($n = 7$; $P < 0{\cdot}01$). Islet cells incubated with the same sera followed by addition of normal human mononuclear cells showed a basal release of $1{\cdot}6 \pm 0{\cdot}11$ and a stimulated release of $1{\cdot}46 \pm 0{\cdot}14$ ($n = 7$; ns).

When control sera were used in the absence of mononuclear cells the basal release was $1{\cdot}92 \pm 0{\cdot}3$ and the stimulated release was $3{\cdot}35 \pm 0{\cdot}5$ ($n = 4$; $P < 0{\cdot}01$); when mononuclear cells were added the basal release was $1{\cdot}37 \pm 0{\cdot}38$ and a significantly higher stimulated release occurred $2{\cdot}6 \pm 0{\cdot}38$ ($n = 4$; $P < 0{\cdot}05$) (Fig. 3).

DISCUSSION

Using an *in vitro* test previously described (Debray-Sachs *et al.* 1978) the present work gives evidence that spontaneously diabetic mice of the C57Bl/KsJ db/db strain present both cell-mediated and humoral anti-pancreatic immunity.

T cell-mediated immunity
The stimulated insulin release from murine islet cells is suppressed following incubation with spleen cells from diabetic mice. This inhibition of insulin release is no longer observed following treatment

of spleen cells with monoclonal anti-mouse Thy 1.2 antibody plus complement. This suggests that T lymphocytes are responsible for the above anti-pancreatic activity.

The major histocompatibility complex (MHC) is apparently not involved in this cellular anti-pancreatic process. Lymphocytes from diabetic mice (H-2^d) suppress insulin release from H-2^k and ^{b} as well as H-2^d mouse islet cells and even from heterologous rat islet cells. This is evidence that the antigen involved is organ specific and that the cytotoxic phenomenon is not H2-restricted.

Humoral immunity

Humoral immunity against β cells is associated with this cell mediated immunity. Sera from db/db mice suppress insulin release from isolated islets in the presence of complement and also in the presence of normal human mononuclear cells (ADCC).

Comparison with human diabetics

These observations appear very similar to recent data obtained in the human with lymphocytes from insulin-dependent diabetics (IDD). In such patients, Boitard *et al.* (1982) found inhibition of β cell responses to glucose + theophylline by T lymphocytes. The serum of insulin-dependent diabetic patients also contains a complement fixing IgG which suppresses insulin release from mouse and human islets *in vitro* (Saï *et al.*, 1981). Antibody-dependent cellular cytotoxicity may be present in these IDD patients (Sensi *et al.*, 1981). Thus our observations in mice are somewhat similar to those made in human diabetes. Major differences, however, are the long lasting hyperinsulinaemia in mice and the absence of early morphological damage in islets. β cell necrosis occurs late in the course of this murine diabetes (Baetens *et al.*, 1978), in contrast with the early appearance of the insulitis demonstrated in human (Gepts, 1965) and rat (Like *et al.*, 1979) insulin-dependent diabetes.

Respective timing of autoimmunity and appearance of diabetes

The study of this mouse model was partly undertaken to clarify the following point: does anti-pancreatic immunity precede or follow the appearance of diabetes? The answer to this question may, in effect, be important in discussing the possible role of the immune process in the development of the disease. Our findings show that: (a) anti-pancreatic immunity is found at our earliest test, i.e., at the 10th day of life; (b) no hyperglycaemia was detected at that time, but only at 1 month and (c) hyperinsulinaemia already exists at the 10th day of life. It could be argued that normoglycaemia in the presence of hyperinsulinaemia may in fact be evidence of insulin resistance. In any case, our results show that it is impossible to clearly dissociate the appearance of diabetes and anti-pancreatic immunity, even in the first month of life. This remains compatible with a causal role of the immune process, but does not prove it.

Further research should also try to explain the relationship of anti-pancreatic immunity with hyperinsulinaemia at the beginning of life, in the absence of hyperglycaemia. We have already demonstrated that no anti-insulin immunity can be found (unpublished results). It may be interesting to see whether the anti-β cell immunity might cause primary insulin hypersecretion.

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