Accelerated diabetes in non-obese diabetic (NOD) mice differing in incidence of spontaneous disease

A. G. BAXTER & T. E. MANDEL Walter & Eliza Hall Institute of Medical Research, Parkville, Australia

(Accepted for publication 28 March 1991)

SUMMARY

The NOD mouse is an established model of autoimmune diabetes mellitus. Various lines of NOD mice differ in their incidence of spontaneous diabetes, e.g. 93% of female NOD/Lt mice compared with 46% of female NOD/Wehi mice develop diabetes by 250 days. These two lines were studied under conditions which greatly accelerate the onset of hyperglycaemia. It was hoped that their responses to these manipulations would reveal characteristic differences which would increase our understanding of diabetes resistance in the low incidence NOD/Wehi line. One dose of 300 mg/kg of cyclophosphamide (CP) produced hyperglycaemia in 50% of NOD mice within 2 weeks in both lines. They were also equally susceptible to diabetes induced by splenocyte transfer at 21 days of age from prediabetic 150-day-old NOD/Lt or NOD/Wehi females. Five daily 40 mg/kg doses of streptozotocin (STZ) resulted in a severity of diabetes in the NOD mice greater than in C57BL or SJL/J mice. While the incidence and severity of diabetes induced in the two NOD lines were similar, this appeared to be principally due to sensitivity to the toxic effects of STZ rather than its ability to exacerbate autoimmune β cell destruction. It has previously been shown that it is possible to prevent diabetes in susceptible NOD mice with simple, relatively benign therapies and here we show that it is possible to induce diabetes in resistant animals at a rate indistinguishable from fully predisposed individuals. It therefore appears that the prediabetic NOD mouse is poised in an immunologically precarious state with the onset of disease being highly dependent on factors which exacerbate or moderate autoimmune destruction.

Keywords cyclophosphamide streptozotocin cell transfer comparative immunology disease susceptibility

INTRODUCTION

NOD mice were derived from a single diabetic female JCL:ICR outbred mouse which was found to be of low weight, polyuric, polydipsic and glycosuric (Makino et al., 1980). Since their description in 1980, many colonies have been established throughout the world, and maintained in separate breeding programmes. In addition to this potential genetic heterogeneity, NOD mice are profoundly affected by environmental variables such as diet (Elliott, et al., 1988; Scott, Daneman & Martin, 1988; Coleman, Kuzava & Leiter 1990) and exposure to pathogens (Oldstone, 1988; Leiter, 1990). As a result, the incidence of diabetes in different lines varies greatly (Colony Information Sheets, 1989). Two such lines are maintained in the same environment at the Walter and Eliza Hall Institute (WEHI) (Baxter, Adams & Mandel, 1989). This allows the direct comparison of immunological parameters mediating the relative resistance to diabetes in the lower incidence line.

The NOD/Lt line maintains a consistently high incidence of diabetes in females at 250 days (93%), while the NOD/Wehi females become diabetic less frequently (43%). Both lines express a much lower incidence of disease in males (21% versus 6%). Histological examination of islets reveals significantly more severe mononuclear cell infiltration in the high incidence (NOD/Lt) line. This supports our premise that the difference in diabetes incidence results from differences in the severity of autoimmune destruction occurring in the two lines (Baxter, Koulmanda & Mandel, 1991).

To define further the immunological differences between the NOD/Lt and NOD/Wehi lines, their responses to three mechanisms of accelerated autoimmune destruction were examined. These mechanisms were: (i) the ablation of suppressor activity with cyclophosphamide (CP); (ii) the passive transfer of autoreactive lymphocytes; and (iii) the increased exposure of putative autoantigens by the repeated administration of subdiabetogenic doses of the β cell toxin, streptozotocin (STZ).
MATERIALS AND METHODS

Mice
NOD/Wehi mice were obtained from the colony derived from breeding pairs provided by H. Asamoto (Kyoto, Japan) and maintained at the WEHI since May 1984 (21 generations). NOD/Lt mice were obtained from the colony derived from breeding pairs obtained from Dr E. H. Leiter (Jackson Laboratory, Bar Harbour, MN) in January 1987 and maintained at the WEHI for 10 generations. C57BL/KaWehi (F43), C57BL/6JWehi (F58), and SJL/JWehi (F65) mice were obtained from the animal breeding facilities of the WEHI. All mice were allowed free access to food (Barstock mouse pellets, VIC.) and acidified water.

Cyclophosphamide treatment
NOD mice 110 days old were bled for random serum glucose estimation and weighed before i.p. injection of 300 mg/kg pure crystalline CP (Pharmamala, Italy) dissolved in 250 μl phosphate-buffered saline (PBS) or PBS alone. Mice were bled 14 days later, killed and their pancreata removed for histological examination.

Splenocyte transfer
Spleens were harvested under sterile conditions from non-diabetic 150-day-old female NOD mice. A single cell suspension was prepared and red cells lysed in 0·168 m NH4Cl at 37°C. The cells were then washed and resuspended in serum-free PBS. Splenocytes (2·0 × 107 in 100 μl) were injected into the retro-orbital plexus of 21-day-old weanlings under an operating microscope. Control mice were either un.injected or injected with 2·0 × 107 γ-irradiated (20 Gy) splenocytes. Recipients were followed by 2 weekly serum glucose estimations until 150 days of age.

Multiple low dose streptozotocin treatment
Mice 9 weeks of age were injected intraperitoneally with 40 mg/kg STZ (Sigma, MO) dissolved in 250 μl PBS daily for 5 days. The treated mice were bled for serum glucose estimations at 5-day intervals for 30 days and then killed and their pancrea removed for histological examination.

Serum glucose measurement
Each mouse was bled by retro-orbital vein puncture of 100–150 μl and the serum glucose concentration measured by the glucose oxidase technique with a Beckman Glucose Analyzer (Beckman, Fullerton, CA). A mouse was declared diabetic if it was found to have a random serum glucose level >12·0 mmol/l followed by either rapid demise or further values >12·0 mmol/l on subsequent bleeds.

Histological assessment of insulitis
Pancreata were fixed in Bouins’ fixative, transferred to alcohol, paraffin embedded and sectioned at three levels. Slides were stained with haematoxylin and eosin or Gomori aldehyde fuchsin, and independently scored by two observers as previously described (Baxter et al., 1991). Briefly, a semi-objective score from 0 to 4 was assigned to each islet. A score of 0 indicated an islet free of pathology, and a score of 4 indicated virtually complete destruction. The scores were then totalled and expressed as a percentage of the total possible score.

Table 1. The result of injecting 11 NOD/Lt and 15 NOD/Wehi non-diabetic 110-day-old female mice with 300 mg/kg CP compared with control PBS-injected mice. There was no significant difference between the proportions of mice which died or became diabetic in the treated groups (Fisher’s exact test).

<table>
<thead>
<tr>
<th>Line</th>
<th>Treatment</th>
<th>Dead</th>
<th>Diabetic</th>
<th>Non-diabetic</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD/Lt</td>
<td>Cyclo</td>
<td>1</td>
<td>5</td>
<td>5</td>
<td>11</td>
</tr>
<tr>
<td>NOD/Lt</td>
<td>Saline</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>NOD/Wehi</td>
<td>Cyclo</td>
<td>1</td>
<td>7</td>
<td>7</td>
<td>15</td>
</tr>
<tr>
<td>NOD/Wehi</td>
<td>Saline</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

Statistical analysis
Comparison of cumulative incidence of diabetes was made using the χ² test. Yates’ correction was applied if the expected frequency of a cell was <10·0 and the Fisher’s exact probability test was applied if the expected frequency of a cell was <5·0. It was assumed that weight and serum glucose maintained a normal distribution, and Student’s t-test was applied. In the case of hyperglycaemia induced by STZ, the distribution of values was clearly non-normal, and the rank sum test was applied. It was assumed that the insulitis scores of pancreata maintained the same distribution and the rank sum or Kruskal–Wallis tests were applied.

RESULTS

Cyclophosphamide treatment
Twenty-three NOD/Wehi and 23 NOD/Lt 110-day-old female mice were weighed and bled before CP treatment. Six NOD/Lt mice were already diabetic and were excluded from analysis. NOD/Wehi mice weighed an average of 23·3 g (s.d. 1·2 g) while NOD/Lt mice weighted 25·3 ± 1·0 g (P < 0·001, Student’s t-test). There was no significant difference between the pretreatment serum glucose estimations. Fifteen NOD/Wehi and 11 NOD/Lt mice were injected with 300 mg/kg CP and the remaining mice were injected with PBS. Two weeks after injection, one mouse from each treatment group had died, and one-half of the remaining mice in each treatment group were diabetic (Table 1).

To confirm the validity of this result a retrospective analysis of CP-treated control groups from previous experiments completed within the laboratory over the previous 3 years was carried out. Of 114 NOD/Wehi females aged 103–145 days 53% became diabetic compared with 52% of 31 NOD/Lt female mice aged 100–127 days when injected with 300–350 mg/kg CP (not significant, χ² test).

Splenocyte transfer
Transfer of 2·0 × 10⁷ splenocytes from 150-day-old non-diabetic female NOD/Lt mice to 21-day-old female weanlings resulted in an increase in the rate of diabetes onset in both lines (Fig. 1). While NOD/Wehi mice initially appear to lag behind the NOD/Lt line, this difference was not significant (11/21 (52%) versus 3/14 (21%) at 100 days; χ² test). Significantly more NOD/Lt weanlings injected with NOD/Lt splenocytes became diabetic by 150 days than either un injected mice (P < 0·01, χ² test) or those injected with irradiated (20 Gy) splenocytes from 150-day-old non-diabetic female NOD/Lt mice (P < 0·001, χ² test with
Table 2. Weanlings were injected with splenocytes from 150-day-old female NOD/Lt or NOD/Wehi mice, with irradiated splenocytes, or un.injected. The incidence of diabetes in each group at 150 days was compared using the $\chi^2$ test or Fisher's exact probability test, as appropriate.

<table>
<thead>
<tr>
<th>Group</th>
<th>Donor</th>
<th>Recipient</th>
<th>Treatment</th>
<th>Diabetic/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>NOD/Lt</td>
<td>NOD/Lt</td>
<td>None</td>
<td>16/20 (80)*</td>
</tr>
<tr>
<td>B</td>
<td>NOD/Wehi</td>
<td>NOD/Lt</td>
<td>None</td>
<td>10/13 (77)</td>
</tr>
<tr>
<td>C</td>
<td>NOD/Lt</td>
<td>NOD/Lt</td>
<td>20 Gy</td>
<td>2/13 (15)</td>
</tr>
<tr>
<td>D</td>
<td>None</td>
<td>NOD/Lt</td>
<td>None</td>
<td>23/50 (46)</td>
</tr>
<tr>
<td>E</td>
<td>NOD/Lt</td>
<td>NOD/Wehi</td>
<td>None</td>
<td>9/12 (75)</td>
</tr>
<tr>
<td>F</td>
<td>NOD/Wehi</td>
<td>NOD/Wehi</td>
<td>None</td>
<td>6/8 (75)</td>
</tr>
<tr>
<td>G</td>
<td>NOD/Lt</td>
<td>NOD/Wehi</td>
<td>20 Gy</td>
<td>2/12 (17)</td>
</tr>
<tr>
<td>H</td>
<td>None</td>
<td>NOD/Wehi</td>
<td>None</td>
<td>7/50 (14)</td>
</tr>
</tbody>
</table>

Statistical analysis: Groups A and B, Fisher's exact test (NS); Groups A and C, $\chi^2$ test ($P < 0.01$); Groups A and D, $\chi^2$ test ($P < 0.01$); Groups C and D, $\chi^2$ with Yates' correction (NS); Groups E and F, Fisher's exact test (NS); Groups E and G, $\chi^2$ with Yates' correction ($P < 0.02$); Groups E and H, Fisher's exact test ($P < 0.001$); Groups G and H, $\chi^2$ test (NS); Groups A and E, $\chi^2$ test (NS).

* Percentages in parentheses.

Yates' correction, Table 2). Significantly more NOD/Wehi weanlings injected with NOD/Lt splenocytes became diabetic by 150 days than either un.injected mice ($P < 0.0001$, Fisher's exact probability test) or those injected with irradiated splenocytes ($P < 0.02$, $\chi^2$ test with Yates' correction, Table 2). There was no significant difference between the proportion of injected NOD/Wehi and NOD/Lt weanlings which became diabetic ($\chi^2$ test, Table 2).

Curiously, the injection of irradiated splenocytes appeared to lower the proportion of NOD/Lt diabetics. This effect was not great enough to be of statistical significance ($\chi^2$ with Yates' correction).

Splenocytes harvested from 150-day-old non-diabetic female NOD/Wehi mice were equally capable of inducing accelerated diabetes in this model, as there was no significant difference between the proportions of either NOD/Lt or NOD/Wehi weanlings which became diabetic by 150 days induced by NOD/Wehi splenocytes and the proportions which became diabetic after injection of NOD/Lt splenocytes (Fisher's test, Table 2). There was no significant difference in the intensity of lymphocytic infiltration in the pancreata of the experimental groups (Kruskal–Wallis test).

Larger numbers of splenocytes injected did not further accelerate disease onset as the injection of $2 \times 10^7$ NOD/Lt splenocytes together with $2 \times 10^7$ NOD/Wehi splenocytes resulted in the same proportion of diabetic mice as NOD/Lt splenocytes alone (data not shown).

Low dose streptozotocin
Six NOD/Lt, six C57BL/KaWehi and six C57BL/6JWehi 9-week-old male mice were injected daily for 5 days with STZ and bled at 5-day intervals for 30 days. All mice except one C57BL/KaWehi mouse became diabetic. The serum glucose was significantly higher in the NOD/Lt mice than either the SJL/JWehi ($P < 0.005$) or the C57BL/KaWehi ($P < 0.002$, rank sum test) mice. Lymphocytic infiltration and $\beta$ cell destruction was greatest in the NOD/Lt mice and least in the C57BL/KaWehi mice (data not shown).

Six NOD/Lt, six C57BL/6JWehi and six SJL/JWehi 9-week-old female mice were treated similarly. Three NOD/Lt and four SJL/JWehi mice became mildly diabetic while no C57BL/6JWehi mice did (not significant, Fisher's test). The serum glucose levels of NOD/Lt (9.2–21.8, mean 14.2 mmol/l) and SJL/JWehi (9.5–13.6, mean 13.1 mmol/l) mice were not significantly different from each other but were from C57BL/6JWehi (5.7–11.8, mean 9.7 mmol/l) mice ($P < 0.05$, rank sum test).

Twenty-four NOD/Wehi and 23 NOD/Lt male 9-week-old mice were weighed and bled before multiple low dose STZ treatment. There was no significant difference between the weights or pretreatment serum glucose estimations (Student's $t$-test). All NOD/Wehi mice and 19 out of 23 NOD/Lt mice became diabetic ($P < 0.05$, $\chi^2$ test), but this is not likely to be of real significance as the distribution of serum glucose estimations in both groups was similar and bimodal (Fig. 2). Eight of 24 NOD/Wehi and 9 out of 23 NOD/Lt mice fell into a 'low responding' group with serum glucose ranging from 9.4 to 25.0 mmol/l. The remainder were 'high responders', with the majority having serum glucose estimations in excess of 40.0 mmol/l. There was no significant difference in the severity of hyperglycaemia between the two lines (rank sum test).

**DISCUSSION**

In this paper we have shown that two lines of NOD mice which differ in their spontaneous incidence of diabetes respond similarly to three different mechanisms of accelerated disease.
Accelerated diabetes in NOD mice

Splenocytes from overtly diabetic NOD mice can transfer diabetes to irradiated adult (Wicker, Miller & Mullen, 1986) or unirradiated neonatal (Bendelac et al., 1987) syngeneic mice. This property is also found in the splenocytes of non-diabetic NOD mice older than 50 days, and is maximal in female mice older than 130 days (Bendelac et al., 1987), appearing to parallel in vitro reactivity to islets. Disease transfer is dependent on recipient and donor age and sex, and the number of cells transferred.

It was hoped that this model would allow differentiation between the activity of autoreactive T cells present in the splenocyte inoculum and the resistance to transfer in the recipient. The finding that both lines were equally susceptible to induction of diabetes by NOD/Lt splenocytes suggested that NOD/Wehi mice do not have a stronger 'suppressor' of immune destruction than NOD/Lt mice, but rather that they simply undergo a less vigorous autoimmune activation. However, this hypothesis must also be discarded in view of the finding that NOD/Wehi splenocytes were equally capable of transferring disease. Thus, it is unlikely that the model suggested by Reich et al. (1989a, 1989b) of two opposing populations of lymphocytes, one autoreactive and the other regulatory, provides a satisfactory explanation for the processes involved in this model.

STZ is a broad spectrum antibiotic with diabetogenic properties mediated by direct β cell cytotoxicity (Rakieten, Rakieten & Nadkarni, 1963). Five daily i.p. injections of a subdiabetogenic dose (40 mg/kg) of STZ were found to result in lymphocytic infiltration of the islets of Langerhans, autoimmune β cell destruction and subsequent hyperglycaemia in permisive strains (Like & Rossini, 1976). A regime of six 35 mg/kg doses resulted in severe hyperglycaemia in males, but only moderate hyperglycaemia in females despite inducing a similar degree of lymphocytic infiltration. Furthermore, male athymic mice were also susceptible to severe hyperglycaemia despite the absence of an islet infiltrate (Leiter, 1982). Thus diabetes induced by the multi-low dose STZ protocol appears to involve the dual action of incremental toxic destruction and the induction of autoimmunity to a β cell neoantigen (reviewed in Kolb, 1987). One possible source of β cell associated neoantigen is C-type retrovirus-like particles which were noted to be dramatically increased in the β cells of STZ-treated mice (Like & Rossini, 1976). Similar particles were seen in the cisternae of the rough endoplasmic reticulum of prediabetic NOD mice (Fujino-Kurihara et al., 1985) and were greatly increased following treatment with CP (Suenaga & Yoon, 1988), suggesting a common, retroviral, autoantigen in all three models.

Thus three possible mechanisms of β cell destruction potentially occur in the low dose STZ model: incremental toxicity, exposure and activation by novel STZ-induced antigen(s), and increased exposure to an existing, stimulatory autoantigen. The finding that it is unable to induce severe hyperglycaemia in female NOD/Lt mice suggests that the third mechanism does not play a role as there does not appear to be any evidence of a primed response in mice that were undergoing active autoimmune destruction of β cells at the time of treatment. The two NOD lines appeared to be equally susceptible to both the toxic and autoimmune mechanisms.

It has previously been shown that it is possible to prevent diabetes in susceptible NOD mice by manipulation of environment (Williams et al., 1990), diet (Elliott et al., 1988; Scott et al.,

Fig. 2. NOD/Lt and NOD/Wehi male mice were injected with five daily doses of 40 mg/kg streptozotocin. Serum glucose estimations were performed on the 30th day after the first injection. The severity of hyperglycaemia is shown as a histogram demonstrating the proportion of mice in each line with plasma glucose levels within each range. Proportions are indicated as the probability a mouse will fall within each given range. A bimodal response was seen in both lines.

Previous studies have addressed the issue of acceleration of diabetes in NOD mice. Harada & Makino (1984) administered two 150 mg/kg doses of CP 2 weeks apart resulting in acute onset of diabetes in the majority of NOD mice. This response was maximal between the ages of 50 and 140 days. A single injection of a higher dose (200–350 mg/kg) (Yasunami & Bach, 1988; Charlton et al., 1989) was also effective, and the proportion of animals rendered diabetic appeared to be dose-dependent (Charlton et al., 1989). Splenocytes from animals rendered diabetic in this manner were able to transfer disease to naive recipients, indicating the mechanism of action was not β cell toxicity (Yasunami & Bach, 1988). Animals injected with CP could be 'rescued' from diabetes onset by multiple early injections of spleen and lymph node cells, suggesting that the acceleration of diabetes resulted from an imbalance in a lymphoid compartment (Charlton et al., 1989). This imbalance has been postulated to be a selective depletion of cells which mediate immunological suppression, as CP has a similar action in the induction of experimental allergic encephalomyelitis (Lando, Teitelbaum & Arnon, 1980) and autoimmune diabetes induced by multiple low dose STZ (Keisel et al., 1981).

Here we have shown the NOD/Wehi and NOD/Lt lines were equally susceptible to CP-precipitated diabetes despite differing in their incidence of spontaneous disease. If the action of CP in this model is the selective depletion of suppression, this result can be explained by supposing an increased level of suppression in the NOD/Wehi line which normally reduces the incidence of diabetes, but which is eradicated by CP treatment.

Acceleration of diabetes by splenocyte injection has been studied in detail by several groups. Splenic T cells from NOD mice respond to islet antigens in vitro by proliferation and the production of IL-2. This response is first seen in 50-day-old mice, and is maximal at 150 days (Nagata et al., 1989).
1988; Coleman et al., 1990) and exposure to pathogens (Oldstone, 1988; Leiter, 1990). Here we show that it is possible to induce diabetes in relatively resistant animals at a rate indistinguishable from fully predisposed individuals. These experiments throw little light on the mechanism of resistance to diabetes in the NOD/Wehi mice. While the CP data are consistent with an increased level of suppressor activity, the cell transfer experiments do not support this hypothesis. Paradoxically, the cell transfer experiments are not consistent with a less aggressive autoimmune attack either. The STZ experiments fail to resolve this problem, as the mechanism of action appears not to be related to the pre-existing autoimmune destruction occurring in the NOD. It is clear, however, that regardless of the mechanism of resistance in the NOD/Wehi line, it is easily overcome by experimental manipulation. It therefore appears that the prediabetic NOD mouse is poised in an immunologically precarious state with the onset of disease being highly dependent on factors which exacerbate or moderate autoimmune destruction.

ACKNOWLEDGMENTS

This work was generously supported by Hoechst Australia Pty Ltd., NH&MRC, JB Were and Sons, The Warman Trust and The Perpetual Trustees and Executors Association of Australia. A.G.B. receives a Hoechst postgraduate research scholarship. We would like to thank M. Koulmanda and Qiaoling Huang for technical assistance; A. Szalai for assistance with animal care; and S. Mihaljovic and T. Bucci for preparation of histological sections.

REFERENCES


