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Absence of H-2 Genetic Influence on Streptozotocin-Induced Diabetes in Mice

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Summary. Five daily injections of streptozotocin (40 mg/kg) produced a delayed but progressively increasing level of hyperglycaemia in long term studies with male Naval Medical Research Institute mice and C3D2Fl (DBA 2 J male × C3H/Tif female) F1 hybrid mice. The development of hyperglycaemia was paralleled by decreased amounts of pancreatic immunoreactive insulin as well as degranulation and necrosis of pancreatic B cells. Insulitis was found from days 9–25 after the first injection of streptozotocin. Compared with the F1 hybrid strain the parental inbred strains DBA 2 J and C3H/Tif demonstrated a certain resistance to streptozotocin. Development of hyperglycaemia did not differ in four congenic resistant lines of mice on the C57 BL/10 genetic background, indicating that major histocompatibility complex genes are not likely to determine susceptibility to streptozotocin-induced islet B cell damage.

Key words: Mouse, experimental diabetes, streptozotocin, H-2 system, sex hormone, insulitis.

Multiple doses of streptozotocin (SZ) [1, 2] produce a delayed but progressively increasing state of hyperglycaemia in mice accompanied by lymphocytic infiltration in and around the islets of Langerhans [3]. This resembles the inflammatory lesion observed in newly diagnosed Type 1 (insulin-dependent) diabetes mellitus [4, 5].

Whereas HLA-linked genes influence the susceptibility to develop Type 1 diabetes [6, 7], an influence of the major mouse histocompatibility complex, H-2 on SZ-induced diabetes mellitus, has been reported not to occur [8]. Thus various inbred strains of mice sharing the same H-2 haplotype demonstrated quite different degrees of hyperglycaemia and insulitis following the same type of treatment with low doses of SZ. However, it is difficult to interpret these data, since genes other than major histocompatibility genes might also be involved in the production of diabetes after SZ treatment, thereby masking a possible H-2 influence. Recently, a modulating effect of H-2 genes on the levels of hyperglycaemia observed in SZ-treated mice was reported [9]. In order to illucidate the genetic influence in further detail, we have studied different strains of mice, including an F1 hybrid strain and congenic resistant lines sharing the background genome but with different H-2 haplotypes [10].

Material and Methods

Ten-week old male or female mice were used in all experiments and also a group of 6 month-old animals. The animals were caged in groups of three to five with free access to water and standard mouse chow (Rostock mixture, Korn og Foderstof Kompagniet, Viby, Denmark). NMRI Bom (Naval Medical Research Institute) mice and the inbred strains DBA/2J (H-2^d), C3H/Tif (H-2^k) and C3D2F1 (DBA/2J ♂ × C3H/Tif ♀) were purchased from Gl. Bomholtgård, Ry, Denmark. C57 BL/10 Sn (H-2^b) and the congenic resistant lines derived from this strain B10. D2/n Sn (H-2^d), B10.A/Sg Sn (H-2^a) and B10.BR/Sg Sn (H-2^k) were obtained from the Jackson Laboratories, Bar Harbor, Maine, USA.

On five consecutive days the animals were given between 08.00 and 10.00 h IP injections of SZ (40 mg/kg-Lot No. U 9889-Upjohn, Kalamazoo, Michigan, USA)) dissolved in saline solution (10 g SZ/l) immediately before the administration. Control animals received only saline solution. Random blood glucose concentrations [11] were measured between 09.00 and 10.00 h in samples from the retro-orbital venous plexus. Immunoreactive pancreatic insulin was determined by radioimmunoassay [12] from 20% acetic acid extract of whole pancreas [13], using crystalline mouse insulin (Novo, Copenhagen, Denmark) as standard. Extracts were appropriately diluted and read in the standard curve between 500 and 2000 pg/ml. Lower detection limit was 70 pg/ml. DNA was determined by the method of Kissane and Robins [14].

Before treatment with SZ or saline solution, animals were selected for morphological investigation during the course of investigation (0–140 days). After sacrifice by decapitation, pancreases were carefully removed, fixed in phosphate-buffered 4% formalin and embedded in paraffin. Sections were stained with haematoxylin-eosin or alde-

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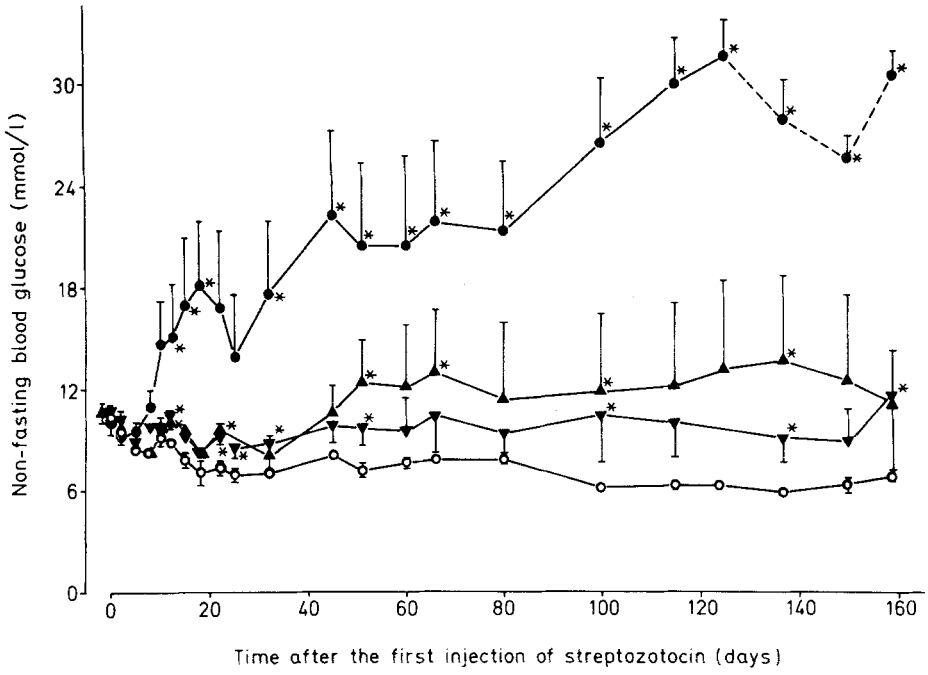

Fig. 1. Non-fasting blood glucose values (mean ± SEM) in 10 week old male NMRI mice following five daily injections of streptozotocin (40 mg/kg, ●), streptozotocin (30 mg/kg, ▲), streptozotocin (20 mg/kg, ▼) or saline solution (○). Each point represents five animals, except on three occasions when only three surviving animals were tested. These values are connected by a dotted line. Streptozotocin injected animals demonstrated higher blood glucose values than saline injected: * ($p < 0.02$), (Wilcoxon's two sample location test)

hyde-fuchsin [15] or were silver-impregnated [16]. For each pancreas all of the 10 randomly selected islets were arbitrarily scored 0–4+ for (1) architectural disarray and lymphocytic infiltration, (2) depletion of aldehyde-fuchsin staining and (3) appearance of silver stained granules, 0 indicating normal appearance, 1+ to 4+ indicating increasing changes. A range of 0–40 scores per pancreas was thus obtained. The morphological preparations were evaluated on coded slides by one to two investigators.

Statistical analysis: Individual or mean values ± SEM are shown throughout for the number of animals indicated. Non-parametric statistical methods were used to calculate levels of significance.

Results

Five daily injections of SZ (40 mg/kg) produced a delayed but progressively increasing degree of hyperglycaemia in male NMRI mice followed for 159 days (Fig. 1). Two animals died 120 days after the first injection of SZ, these animals being the first to develop hyperglycaemia. The figure also shows that SZ (20 or 30 mg/kg) had little effect on blood glucose.

A similar pattern of progressively increasing hyperglycaemia was observed in C3D2F1 male mice (not shown). Extractable pancreatic immunoreactive insulin declined from 66 ± 5 μg ($n = 4$) before SZ treatment to 30 ± 3 μg ($n = 4$) at day 7, 23 ± 3 μg ($n = 4$) at day 20 and to values below 5 μg from day 48 following the first SZ injection. Pancreatic insulin content was significantly reduced compared with the controls, from day 10 to the end of the experiment 140 days after the first injection of SZ or saline solution ($p < 0.03$; Wilcoxon's two sample location test). Pancreatic DNA did not differ from controls on day 10 after the first injection of SZ (754 ± 24, $n = 8$ versus 723 ± 16 pg, $n = 8$) and day 23 (935 ± 54, $n = 8$ versus 963 ± 51 pg, $n = 10$).

Morphological examination of the F1 hybrid animals revealed signs of increasing degree of necrosis after SZ treatment. Thus scores from individual pancreases increased from 5–10 before SZ treatment to 15–30 days 75–140 following the first injection. The slope coefficient for necrosis – scores for SZ injected animals ($n = 27$) was greater than zero ($p < 0.01$; a distribution-free test for the slope coefficient (Theil)), but not for the control animals ($n = 14$; $p > 0.11$). Whereas insulitis with lymphocytic infiltration was seen only from days 9–25 after the first injection of SZ, architectural disarray was most marked after 50–70 days. The SZ treatment resulted in a decrease in aldehyde-fuchsin staining most marked after 20–30 days following the first SZ injection. The slope coefficient for insulin-depletion – scores for SZ injected animals ($n = 27$) was greater than zero ($p < 0.001$), but not for the control animals ($n = 14$; $p > 0.36$).

Silver impregnated sections were also prepared at days 10 and 23 after the first injection of SZ. At day 10 an increased amount of silver stained granules was seen in SZ-treated animals compared with the controls (scores: 7 ± 2, $n = 3$ versus 2 ± 1, $n = 3$). This was also true at day 23 (scores: 10 ± 1, $n = 3$ versus 1 ± 0, $n = 3$), which makes the observation of increased silver staining of pancreatic islets after SZ treatment highly significant ($p = 0.002$).

In a third series of experiments, non-fasting blood glucose values were followed for 121 days in 10 week or 6 month old male C3D2F1 mice together with 10 week old male mice of the parental strains DBA/2J and C3H/Tif. The pattern of progressively increasing hyperglycaemia (25–35 mmol/l) in younger F1 hybrid mice was confirmed. However, the two parental strains as well as the elder F1 hybrids demonstrated moderate hypergly-

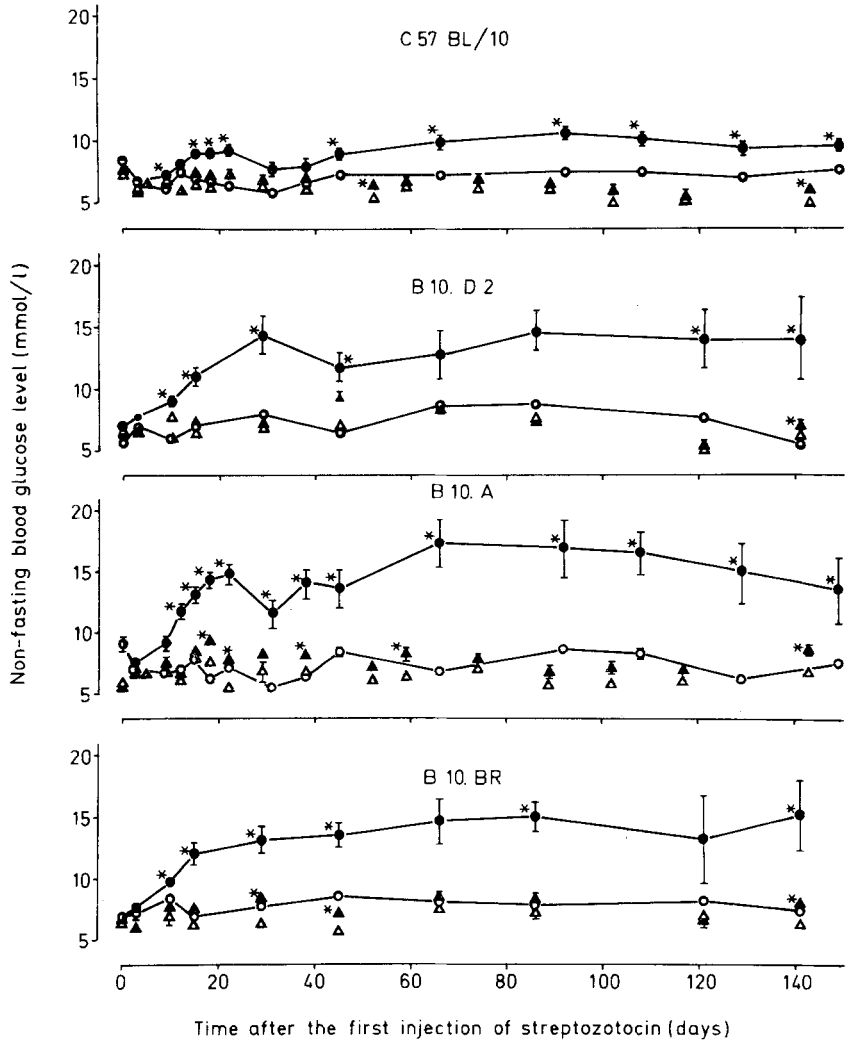

Fig. 2. Non-fasting blood glucose levels in 10 week old male (circles) and females (triangles) C57 BL/10 Sn, B10.D2/n Sn, B10. A/Sg Sn and B10.BR/Sg Sn mice following five daily injections of streptozotocin (40 mg/kg, solid symbols) or saline solution (open symbols). Each point indicates the mean ± SEM values of five animals. Streptozotocin-injected animals demonstrated higher values than controls: * ($p < 0.02$) (Wilcoxon's two sample location test)

Table 1. Non-fasting blood glucose levels following streptozotocin treatment in congenic resistant lines of mice

Mouse strain	H-2	Blood glucose levels (mmol/l)			
		Experiment 1		Experiment 2	
		Control mice	SZ × 5 injections	Control mice	SZ × 5 injections
C57 BL/10 Sn	H-2^b	7.0 ± 0.1 (14)	8.9 ± 0.3 (14)	7.3 ± 0.2 (26)	10.7 ± 0.4 (26)
B10.D2/n Sn	H-2^d	7.2 ± 0.4 (9)	12.1 ± 0.8 (9)	7.5 ± 0.2 (26)	11.4 ± 0.5 (26)
B10.A/Sg Sn	H-2^a	7.1 ± 0.2 (14)	13.5 ± 0.8 (14)	7.1 ± 0.2 (22)	10.1 ± 0.4 (22)
B10.BR/Sg Sn	H-2^k	7.8 ± 0.2 (9)	12.6 ± 0.8 (9)	7.6 ± 0.2 (22)	12.4 ± 0.5 (22)

Results are expressed as mean ± SEM; number of observations given in parentheses, of non-fasting blood glucose levels measured at frequent intervals days 3–148 following the first of five daily injections of saline (control mice) or streptozotocin 40 mg/kg (SZ × 5 injections). Individual values of daily blood glucose for experiment 1 are shown in Figure 2. Experiment 2 was conducted according to the same protocol as experiment 1, the only difference being more frequent estimations of blood glucose. In experiment 1, male C57 BL/10 Sn mice treated with SZ demonstrated a lower level of mean blood glucose values than males from any of the other three strains ($2\,p < 0.01$). In experiment 2 no significant differences in levels of hyperglycaemia were observed in the four strains of mice

caemia only with random blood glucose values of 10–15 mmol/l.

Random blood glucose levels in four congenic resistant lines of mice are shown in Figure 2. After SZ treatment, males from all four strains developed persisting hyperglycaemia. Female animals only occasionally showed smaller elevations of blood glucose. This experiment was repeated using the same strains, number of animals and study period as shown in Figure 2. Both series of experiments are summarized in Table 1, which shows the levels of mean blood glucose measured at frequent intervals 3–148 days after the first injection of SZ or saline solution in H-2^b, H-2^d, H-2^a and H-2^k male animals. Only minor differences between hyperglycaemic

levels were observed, the relative resistance of H-2^b animals shown in Figure 2 not being confirmed in the repeated experiment.

Discussion

Our long term studies indicate that observation periods of at least 3–4 months may be necessary to determine the full effect of low dose SZ treatment.

Blood glucose and pancreatic insulin estimations showed that insulin content declines before the main increase in blood glucose occurs, which is in accordance with other reports [12, 17]. This may reflect a large reserve ability to maintain normoglycaemia and partial pancreatic damage may not be followed by severe diabetes as in our experiments with doses of SZ < 40 mg/kg.

Decreased amount of pancreatic insulin could also be detected by morphological studies, which showed degranulation of islets shortly after the termination of SZ treatment. Insulitis was mainly seen in weeks 1 and 2 after the termination of SZ injections, at which time the main decline in pancreatic insulin content had already occurred, which is in accordance with Bonnevie-Nielsen et al. [12]. Inflammation could not be traced by DNA determinations of whole pancreas. The silver stain for A cells in our experiments looked even more pronounced in islets from SZ-treated animals. Whether this reflects an absolute increase in numbers of A cells or just a relative increase in islets depleted for B cells could not be determined by our qualitative scoring method.

The experimental series with parental mouse strains, which were relatively resistant to SZ treatment in contrast to their F1 hybrids, might favour an epistatic interaction or overdominant mode of inheritance. This should be analyzed further in experiments including F2 hybrid and backcross strains.

Little is known of the possible influence of major histocompatibility complex genes on the susceptibility to SZ treatment. Immune response genes are closely linked to the major histocompatibility complex genes [18], and some influence of H-2 on the degree of experimental autoimmune diabetes has been shown [11]. H-2 did not influence encephalomyocarditis virus-induced experimental diabetes [11, 19]. Rossini et al. compared inbred strains of mice treated with low doses of SZ [8]. Some of these shared the same H-2 haplotype (H-2^d), but demonstrated varying degrees of hyperglycaemia and insulitis. However, these experiments did not exclude an influence of major histocompatibility complex genes, since both extra H-2 linked genes and genes outside the H-2 complex may act in concert by suppressing or enhancing susceptibility to SZ treatment. To investigate this further, we studied congenic resistant lines of mice differing from one another only in the portion of chromosome 17 which includes the H-2 region. However, comparative experiments of this kind are difficult to interpret since either the time of onset of diabetes, level of hyperglycaemia or the rate of progressive increase in blood glucose could be taken as a measure of susceptibility. In addition, glucose removal mechanisms might blur blood glucose levels. Indeed the major histocompatibility complex has recently been demonstrated to influence liver adenylate cyclase activity [20]. In our experiments all four H-2 different strains studied developed significant hyperglycaemia. In one of the two series of longitudinally conducted experiments, the level of hyperglycaemia was lower in C57 BL/10 Sn mice when compared with the other three strains. This could not be confirmed in the second series of experiments, disclosing certain inter-experimental variations. All strains had developed significant hyperglycaemia after 12 days following the first injection of SZ. None of the strains demonstrated progressively increasing blood glucose values. For these reasons we conclude that major histocompatibility complex genes do not influence the susceptibility of male mice to SZ treatment. However, it cannot be excluded that H-2 specificities other than b, d, a or k confer susceptibility. Our conclusion is in variance with that of Kiesel and Kolb [9], who observed different plateaux of blood glucose values in SZ-treated congenic resistant lines of mice. All their strains of mice developed hyperglycaemia compatible with diabetes as in our study, and their conclusion may reflect different interpretation of results. In our study, H-2 genes did not influence the resistance of female animals, since all females appeared resistant.

The resistance of females was considered to reflect different levels of sex hormones [21, 22]. It is not known, however, whether different concentrations of sex hormones in males may influence susceptibility to SZ treatment. Such an effect might be under genetic influence. It should be noted, for example, that testes weights are known to vary considerably in different inbred strains of mice, C57 Bl/10 having the lowest testes weights among 24 strains studied [23]. Our data on decreased susceptibility of aged mice support the concept of a modulating effect of sex hormones. Studies on sex hormone concentrations are clearly indicated and should be included in future studies on the genetics of experimental diabetes.

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