Abnormal lymphocyte function precedes hyperglycaemia in mice treated with multiple low doses of streptozotocin

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Summary. To investigate early immunological disturbances at the onset of diabetes, lymphocyte function and islet cell surface antibodies were studied in streptozotocin-treated C57BL/10 and B10.BR mice. In C57BL/10 mice, streptozotocin given in multiple low doses depressed lymphoblastic transformation to phytohaemagglutinin and pokeweed mitogen, but not to concanavalin A on day 6 after the first administration. On day 20, the transformation remained suppressed with phytohaemagglutinin, but recovered to the control level with pokeweed mitogen. In the early phase after treatment, the islet cell surface antibodies were elevated and then declined. Single high dose administration depressed responses

to phytohaemagglutinin with no detectable islet cell surface antibodies. In B10.BR mice transformations to pokeweed mitogen and concanavalin A were suppressed in the early phase. The strain of mice may be a factor to be considered. Thus, it was suggested that the deterioration of immunological function with the formation of islet cell surface antibodies preceded the onset of hyperglycaemia in mice treated with multiple low doses of streptozotocin.

Key words: Lymphocyte function, experimental diabetes, islet cell surface antibody, streptozotocin, mice, insulinitis.

There are many reports suggesting that autoimmune reactions are involved in the pathogenesis of insulin-dependent diabetes. The presence of islet cell antibodies (ICA) [1–2] or islet cell surface antibodies (ICSA) [3] in an early phase of the disease suggests they may have a pathological role in the onset of diabetes.

Multiple administration of low doses of streptozotocin causes insulinitis in mice which resembles human insulin-dependent diabetes [4], while single injections of high doses of streptozotocin cause degranulation and reduction in number of β cells in the pancreatic islets without insulinitis. To investigate the early immunological disturbances at the onset of insulin-dependent diabetes, lymphocyte function and ICSA were estimated in streptozotocin-treated animals.

Materials and Methods

Seven- to eight-week old male mice (C57BL/10 [H-2^b] and B10.BR [H-2^k]) were caged in groups of three to five with free access to water and standard mouse chow. On five consecutive days mice were given intraperitoneal injections of streptozotocin (40 mg/kg body weight) dissolved in citrate buffer (pH 4.5) immediately before the administration. Single injections of high doses of streptozotocin (200 mg/kg body weight) were administered to another group. Control animals

received citrate buffer only. Treatment with streptozotocin was carried out using 36 C57BL/10 and 29 B10.BR mice, while 32 C57BL/10 and 28 B10.BR were used as controls. Each group was separated into subgroups (Fig. 1, numbers in parentheses). On days 2, 6, 20 and 41 after the first injection of streptozotocin or citrate buffer, spleens and pancreases were removed after collection of blood. Blood glucose was analysed by autoanalyser.

Pancreases were fixed in formalin and embedded in paraffin, followed by staining with haematoxylin-eosin. Spleens were transferred to RPMI-1640 medium containing 10% heat-inactivated fetal calf serum. After gentle homogenization, the tissue was filtered through 150 mesh followed by density gradient with Ficoll-paque. Lymphocytes (200 μ l; 5×10^6 /ml) and 20 μ l of mitogen were incubated for 72 h in a humidified atmosphere containing 5% CO₂ at 37°C. The final concentrations of phytohaemagglutinin (PHA), pokeweed mitogen (PWM), and concanavalin A were 1 μ g/ml, 50 μ g/ml and 5 μ g/ml. The islets and spleen cell homogenates were obtained from Wistar male rats and used at a concentration of 0.1 μ g/ml. [³H]-thymidine (20 μ l) was added 20 h before the harvest. Lymphocytes were solubilized and counted in a scintillator.

ICSA were measured by an indirect immunofluorescence method according to a modified method of Lernmark et al. [3]. Free islet cells (1×10^5) were incubated with 100 μ l of inactivated test serum for 60 min at room temperature. After washing, cells were subsequently incubated with 100 μ l of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG [F(ab')₂] or IgM (μ -chain specific) for 30 min at room temperature. After washing, FITC-positive cells were counted under a fluorescent microscope. Sera which reacted with more than 10% of 50–100 cells were evaluated as ICSA positive.

Statistical analyses were performed with the Student's t-test, χ^2 testing and Wilcoxon's rank sum test.

Fig. 1. Lymphoblastic transformation of spleen cells from C57BL/10 and B10.BR mice treated with streptozotocin. Open (control) or hatched (streptozotocin-treated) columns represent ³H-thymidine (³H-TdR) incorporation by cultured spleen cells in response to PHA, PWM, concanavalin A (Con A) or without any mitogen (-). Small horizontal bars represent the SEM of (n) independent experiments (each in triplicate) on day 4 of culture. The lowest left panel shows the transformation of lymphocytes from C57BL/10 mice treated with a high dose of streptozotocin on day 20 after administration

Results

Non-fasting glucose levels in mice following injections of streptozotocin

In both C57BL/10 and B10.BR mice, blood glucose levels were not elevated on day 6 after five consecutive administrations of multiple low doses of streptozotocin (9.5 ± 1.6 and 9.8 ± 0.9 mmol/l, respectively; control mice 9.9 ± 0.6 and 10.8 ± 0.9 mmol/l), but were significantly elevated on day 20 (18.1 ± 1.1 and 22.7 ± 2.0 mmol/l; control mice 9.6 ± 0.5 and 11.6 ± 0.9 mmol/l, $p < 0.001$). Single high dose injections of streptozotocin caused more prominent hyperglycaemia in C57BL/10 mice (29.5 ± 3.2 mmol/l; control mice 9.5 ± 0.6 mmol/l, $p < 0.001$).

Lymphoblastic transformation of spleen cells (Fig. 1)

The relationship between the day after the first administration of streptozotocin and the ability of lymphocytes to respond to PHA, PWM and concanavalin A is shown in Figure 1. On day 6, C57BL/10 control mice showed an approximately two- two- and fourfold increase of

³H-thymidine incorporation in response to PHA, PWM and concanavalin A, respectively. Mice treated with streptozotocin showed depressed responses to PHA by 40% ($p < 0.001$), and to PWM by 45% ($p < 0.001$) respectively, but did not to concanavalin A. On day 20, the transformation remained suppressed with PHA, but recovered to the control level with PWM. On day 41, the responses to those mitogens were not different between streptozotocin-treated and control mice. With single injections of high doses streptozotocin the responses to PHA were lower than those in control mice by 31% ($p < 0.02$).

In B10.BR mice, the responses of spleen lymphocytes to concanavalin A were low on day 2 and those to PHA were significantly low on day 6. The transformation of PWM in streptozotocin-treatment was the same as in control mice from days 2–41.

Prevalence of positive ICSA (Table 1)

In the control groups of both strains of mice, no detection of ICSA in the sera was seen. A significantly high rate of positive ICSA was observed on day 6 using multiple low doses of streptozotocin in C57BL/10 mice.

Table 1. Prevalence of positive islet cell surface antibodies (ICSA) in two strains of mice

Streptozotocin	Days after the first administration of streptozotocin	C57BL/10 Mice		B10.BR Mice			
		Control	Streptozotocin treated	Control		Streptozotocin treated	
		IgG (%)	IgG (%)	IgG (%)	IgM (%)	IgG (%)	IgM (%)
Multiple low doses (five) (40 mg/kg body weight)	2	0 (0/4)	0 (0/5)	0 (0/4)	—	20 (1/5)	50 (2/4)
	6	0 (0/5)	83 (5/6) ^a	0 (0/6)	25 (1/4)	27 (3/11)	71 (5/7)
	20	0 (0/7)	44 (4/9)	0 (0/5)	—	40 (2/5)	—
	41	0 (0/3)	33 (2/6)	0 (0/5)	0 (0/2)	25 (1/4)	100 (2/2)
Single high dose (200 mg/kg body weight)	20	0 (0/7)	0 (0/6)	—	—	—	—

Values in parentheses denote the number of positive observations out of the total number of samples examined. ^a $p < 0.05$; significance level was assessed using χ^2 test

The positive rate of ICSA declined thereafter. In the case of B10.BR mice, the prevalence of ICSA was high on day 20, although the differences among the days were not significant. In the group of C57BL/10 mice treated with single high doses of streptozotocin, no ICSA were detected. By examining a few indiscriminately chosen samples, IgM and IgG class ICSA were found to have contributed to ICSA elevation shortly after streptozotocin treatment.

Lymphoblastic transformation to specific antigens in B10.BR mice treated with multiple low doses of streptozotocin on day 6

Lymphoblastic transformation to rat islet and splenic cell homogenate were tested in B10.BR mice. The islet homogenate augmented transformation in both streptozotocin-treated and control mice. Those in streptozotocin-treated mice were higher than in control mice, but were not significant. Splenic cell homogenates did not stimulate the transformation.

Discussion

This study demonstrates that impaired lymphoblastic transformation to non-specific mitogens accompanied emergence of ICSA in mice soon after treatment with multiple low doses of streptozotocin. The low responses of spleen lymphocytes to PHA, PWM and concanavalin A were detected before blood glucose levels rose. Therefore the impairment of lymphocyte function cannot be attributed to deterioration in glucose metabolism despite the report that lymphocyte responses were attenuated in poorly controlled diabetes [5]. The depressed response to PHA was also observed in single high doses of streptozotocin, but did not last until day 41, while non-fasting blood glucose levels were high in both cases. The existence of ICSA in multiple low dose treatment but not in single high dose treatment suggested an involvement of autoimmune reactions on

the extension of islet damage following the direct effect of streptozotocin, though the toxicity of streptozotocin has been said to be solely on pancreatic β cells [6]. Additional evidence for autoimmune reactions was the finding of insulinitis in mice with ICSA-positive sera. The occurrence of insulinitis was prevented by administration of anti-lymphocyte sera [7], hydrocortisone [8], or total body irradiation [9], but not by administration of 3-O-methyl-D-glucose [7]. Transfer of lymphocytes from multiple low dose streptozotocin treated to congenic thymusless C57BL/6J-nu/nu mice was reported to induce lymphocytic infiltration of pancreatic islets [10], and it was suggested that low dose streptozotocin treatment induced cellular immune reactions against pancreatic islets. The augmented response of lymphocytes to islet homogenate may suggest such a mechanism. Because complement-dependent cytotoxicity has been reported [11–12], the emergence of ICSA can be postulated as the trigger of further cellular response rather than the result of it.

Whereas HLA-linked genes influence the susceptibility to develop insulin-dependent diabetes, the influence of major histocompatibility complex (H-2) on streptozotocin-induced diabetes in mice is controversial [13–15]. Our data suggest that the development of hyperglycaemia did not differ in two congenic resistant lines of mice of the C57BL/10 genetic background, but lymphoblastic transformations by each mitogen were slightly different between the two lines suggesting different susceptibility of lymphocyte subsets to treatment with multiple low doses streptozotocin.

Thus it might be deterioration in immunological function rather than disturbed glucose metabolism that caused the depressed response of lymphoblastic transformation in the early phase following the treatment with streptozotocin.

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