Assessment of the diabetogenic drugs alloxan and streptozotocin as models for the study of immune defects in diabetic mice

G. N. Gaulton¹, J. L. Schwartz² and D. D. Eardley³

¹Department of Pathology, Harvard Medical School and ²Department of Cancer Biology, Harvard School of Public Health, Boston, Massachusetts, and ³Department of Biological Sciences, University of California Santa Barbara, Santa Barbara, California, USA

Summary. The efficacy of the diabetogenic drugs streptozotocin and alloxan were evaluated as models for the study of immune defects associated with diabetes. Streptozotocin- or alloxan-treated mice, with a stable hyperglycaemia of 25–33 mmol/l plasma glucose, were severely impaired in their ability to mount antibody forming, mitogenic, or delayed-type hypersensitivity responses *in vivo*. Treatment of alloxan-diabetic mice with insulin *in vivo* completely reversed all immune defects, while insulin treatment of streptozotocin-diabetic mice restored immune function to only 70–80% of normal levels. Results obtained by viability measurements and *in vitro* biological assays of lymphoid function, including proliferation in response to T- and B-cell mitogens, the production of interleukin-2 by T cells, and the production of interleukin-1 by macrophages indicated that direct exposure to alloxan for 48 h (at concentrations ≤ 14 mmol/l) had no adverse effects on lymphoid activity, while exposure to streptozotocin was routinely toxic at concentrations ≥ 1 mmol/l. Both alloxan

and streptozotocin exhibited strong toxicity *in vitro* for isolated pancreatic islet cells. Finally, lymphocytes from streptozotocin-diabetic mice, or cells incubated *in vitro* with streptozotocin, contained numerous chromosomal abnormalities indicative of DNA strand breakage. Such abnormalities were absent in alloxan-diabetic mice and in cells incubated with alloxan *in vitro*. These results indicate that immune dysfunction associated with streptozotocin is attributable to direct and irreversible impairment of lymphoid cell function and viability. In contrast, immune dysfunction associated with alloxan-diabetes appears to be a consequence of the diabetic state. Thus, alloxan- but not streptozotocin-diabetes provides a useful model for evaluating immunological changes associated with hyperglycaemia and diabetes.

Key words: Alloxan, streptozotocin, lymphoid toxicity, diabetes, immune function.

Studies conducted over the past 40 years have documented that poorly managed diabetic patients suffer impairment of both cellular and humoral immune responses [1–4]. While the majority of this work accurately describes the general immunodeficiency syndrome, precise mapping of the cells which are primarily affected, and of the relationship between these cells and normal lymphoid cells in the regulation of immune responses, has not been accomplished. In part, these difficulties trace to the lack of a clearly defined murine model for evaluating the immune system of Type 1 (insulin-dependent) diabetic subjects. Both of the genetically linked murine diabetic syndromes (Ob and db) more closely resemble Type 2 (non-insulin-dependent) diabetes in their aetiology. Thus, from the point of view of analyzing altered immunity in the context of the effects of diabetes, these models suffer the added confusion of immune autoreactivity as a possible cause of diabetes [5].

The diabetogenic agents alloxan and streptozotocin (STZ) have provided the most reliable models of Type 1 diabetes in mice [6] and have been widely employed to establish murine models for the analysis of secondary

diabetic phenomena such as altered immune function and ocular pathology. Both drugs exhibit toxicity for B islet cells which is linked to their capacity to accumulate rapidly in these cells and to the high sensitivity of the B cell towards peroxidases relative to other tissues [6–9]. Several studies have reported an impairment of both humoral and cellular immune functions in alloxan- and STZ-diabetic mice [10–13]. However, conflicting reports exist which question the specificity of these diabetogenic agents; most notably, Nevalainen and Hoftiezer [13], and Nichols et al. [14] have detected a direct toxicity of STZ for thymic components of the immune system. These reports have prompted our analysis of the direct effects of STZ and alloxan on the cellular components of the immune system as contrasted to pancreatic islet cells.

Materials and methods

Animals

Inbred DBA/2J female mice (Jackson Laboratories, Bar Harbour, Maine) aged 8–12 weeks, weight 20–25 g were used throughout the experiments. All mice were maintained on Prolab 3000 mouse chow

(Agway, Syracuse, New York) and water ad libitum. Blood glucose levels of nonfasting mice were 6.5–8.6 mmol/l.

Induction and management of diabetes

Mice were rendered diabetic by a single intravenous injection of either alloxan (5,6-dioxyuracil monohydrate, 50 mg/kg body weight) in saline (150 mmol/l NaCl), or STZ (2-deoxy-2-[(methylnitrosoamino)carbonyl]-amino-(D-glucopyranose), 150 mg/kg body weight) in 10 mmol/l citrate buffer (pH 4.5). Mortality was <5%. The metabolic control of diabetic mice was determined by daily weight measurements, measurements of plasma glucose levels, and the levels of pancreatic insulin. Non-fasting whole blood glucose levels were monitored immediately prior to use on days 2 or 5, using the 0-toluidine method [15]. Blood was obtained from the paraorbital venous plexus using heparinized capillary tubes. Only mice which showed no overt signs of diabetes and with plasma glucose levels between 25–33 mmol/l were chosen. Greater than 95% of these mice displayed elevated glucose levels for at least 21 days after drug injection. Mice with glucose levels <20 mmol/l (not used in this study) had a greater than 25% reversion to normal glucose levels by 21 days. Pancreatic insulin levels were determined by radioimmunoassay as described previously [16, 17].

To correlate the doses of alloxan and STZ used *in vivo* to those *in vitro*, an approximation of the concentration of alloxan and STZ in the extracellular fluid compartment has been calculated based on murine fluid compartment size as reported by Bernstein [18], and assuming an unrestricted fluid distribution. For alloxan a 50 mg/kg dose corresponds to a concentration of 1.1 mmol/l, and for STZ 150 mg/kg corresponds to 1.8 mmol/l. The observations of Lee and Dewitt [19], and Weiss [7] indicate that alloxan and STZ accumulate rapidly and preferentially in the pancreatic islets as compared to lymphatic organs and blood. For example, 60 min following intravenous injection the levels of pancreatic alloxan are 96 times those in blood, 78 times those in spleen, and 72 times those in thymus [19]. Thus, the estimated concentrations of these drugs in fluid represent values at maximal levels of potential initial exposure to lymphoid cells.

Where indicated, mice were given 0.33 IU insulin (Sigma, St. Louis, Missouri) by intramuscular injection every 8 h. The metabolic control of insulin-treated mice was determined by weight and plasma glucose measurements, and by glucose tolerance tests. Blood for glucose tolerance tests was obtained 60 min after the intraperitoneal administration of 2 mg of glucose per gram of body weight [17]. Insulin-treated alloxan- and STZ-diabetic mice displayed normal glucose levels after 4 days of management, accompanied by a steady weight gain. Return to weight levels of age-matched normal mice was achieved after 10 days of insulin treatment. Mice shifted off insulin after 4 days of management reverted to previous levels of the diabetic state within 2 days.

Cell isolation

Pancreatic islets were isolated from mice by the collagenase method of Lacy et al. [20] and were maintained in culture in Dulbecco's modified Eagle's Media (Gibco, Grand Island, New York) supplemented with 10% fetal bovine sera, 100 µg penicillin/streptomycin, 4 mmol/l glutamine, 25 mmol/l Hepes (pH 7.2), and 5×10^{-5} mol/l 2-mercaptoethanol at 37 °C in 5% CO₂. Spleen and thymus cells were isolated by sterile excision from freshly killed mice. Organs were washed briefly in RPMI 1640 media (Gibco), supplemented as above then teased apart between two frosted glass slides. Cells were washed three times and harvested by centrifugation at 800 g for 10 min. Splenic macrophages were isolated by the method of Cowing et al. [21] with adherence onto glass Petri dishes for 2 h at 37 °C in supplemented RPMI 1640. Adherent cells were harvested by washing with 1:5000 Versene buffer (Gibco) for 15 min at 37 °C. Cell viability was determined by counting in a Neubauer haemocytometer (Scientific Products, McGraw Park, Illinois) in the presence of 0.04% trypan blue as described by Grankvist et al. [22].

Assays *in vitro*

Anti-sheep red blood cell antibody responses were conducted using the *in vitro* haemolytic plaque assay as modified by Cunningham [23] and Eardley et al. [24]. Spleen cells (1×10^6) in supplemented RPMI 1640 were cultured in 200 µl microtitre wells for 5 days at 37 °C in 5% CO₂ in the presence of 0.05% sheep red blood cells (Pel Freeze, Rodgers, Arkansas). For *in vitro* experiments alloxan or STZ were added to incubations on day 0 or 3 at concentrations between 0.1–14.0 mmol/l. The mean number of plaque forming cells was calculated from triplicate cultures on day 5 and the data are expressed as plaque forming cells/ 10^6 spleen cells plated.

Mitogenic assays were conducted *in vitro* in 200 µl microtitre wells which contained 2×10^5 cells in supplemented RPMI 1640 and either of the mitogens concanavalin A (2.5 µg/ml, Sigma), bacterial lipopolysaccharide (5 µg/ml, Difco, Detroit, Michigan) or phytohaemagglutinin (5 µg/ml, Difco). Alloxan or STZ were added to incubations at the same time as mitogens. Control incubations were conducted in saline (for alloxan) or citrate buffer (for STZ) in the absence of drugs. After 40 h the reactions were pulsed with 1 µCi ³H-thymidine (6.7 Ci/mmol, New England Nuclear, Boston, Massachusetts) and harvested 8 h later. The results of quadruplicate experiments are presented.

Interleukin-2 synthesis was determined in triplicate by incubating spleen cells (2×10^6 cells/ml) in supplemented RPMI 1640 at 37 °C in the presence of 2.5 µg/ml concanavalin A and alloxan or STZ for 48 h. Supernatants were harvested, filter sterilized, and assayed for interleukin-2 by the ability to support the growth of the interleukin-2 dependent line CTLL-2 [25]. CTLL-2 cells (2×10^4 cells in 200 µl) were incubated for 16 h at 37 °C then pulsed with 1 µCi ³H-thymidine and harvested 8 h later.

Whether alloxan or STZ affect interleukin-1 synthesis by splenic macrophages was determined by incubating splenic macrophages with 5 µg/ml lipopolysaccharide at 37 °C in supplemented RPMI 1640, and in the presence of either alloxan or STZ. Supernatants were harvested after 48 h, filtered and assayed for interleukin-1 by the phytohaemagglutinin thymocyte proliferation assay as described previously [26].

Assays *in vivo*

The ability of alloxan or STZ to inhibit cellular immune responses *in vivo* was assessed by examining the delayed-type hypersensitivity response to the hapten azobenzenearsonate as described by Bach et al. [27]. Briefly, mice were immunized subcutaneously with 3×10^7 azobenzenearsonate-coupled syngeneic spleen cells. Five days later mice were challenged in the left foot pad with 33 µl of azobenzenearsonate salt (100 mmol/l). Twenty-four hours later the footpad swelling increment (left foot versus right foot) was determined using an engineer's calipers (Fowler, Neuber, Indiana).

Immunofluorescence staining

To determine the proportions of lymphocyte subpopulations in splenocytes of day 5 alloxan- and STZ-diabetic mice, immunofluorescence studies were conducted. To detect B-cell levels, spleen cell suspensions were stained directly with fluorescein conjugated F(ab')₂ rabbit anti-mouse immunoglobulin (Miles Laboratories, Naperville, Illinois) then fixed in 1% paraformaldehyde. T cell levels were obtained by subtraction of B-cell values from those obtained upon staining of spleen cells with mouse anti-theta 1.2 (monoclonal HO13.4) plus fluoresceinated rabbit anti-mouse immunoglobulin. To measure the proportions of T-cell subclasses, T cells were first purified by panning on goat anti-mouse immunoglobulin (Miles) coated plates and the non-adherent fraction was reacted with either mouse anti-Lyt 1.1 or anti-Lyt 2.1 (a gift of Dr. F. W. Shen, Sloan Kettering Institute, New York, New York) and stained with fluoresceinated rabbit anti-mouse immunoglobulin. For all reactions 1×10^6 cells were incubated with 100 µl of the appropriate diluted antibody at 4 °C for 45 min, washed three times in ice cold balanced salt solution containing 2% fetal bovine se-

ra and 0.1% NaN₃, and then incubated at 4°C for 45 min with fluoresceinated rabbit anti-mouse immunoglobulin. Cells were fixed in paraformaldehyde prior to analysis on a fluorescence activated cell sorter.

Lymphocyte karyotype analysis

Analysis of lymphocyte karyotype was performed on lymphocytes (5×10^6 cells/ml) which had first been incubated for 48 h in supplemented RPMI 1640 in the presence of concanavalin A (2.5 µg/ml) and lipopolysaccharide (5 µg/ml) at 37°C. During the final 2.5 h of this incubation, cells were adjusted to colcemid (2×10^{-7} mol/l) to fix the mitotic apparatus. Cells were harvested and 2 ml of KCl (75 mmol/l) was added drop-wise, and then incubated at 37°C for 20 min. Cells were next pelleted and 2 ml of a freshly prepared fixative (3:1, absolute methanol:glacial acetic acid) was added. Incubation was continued for 15 min at 4°C after which cells were pelleted, resuspended in a minimum volume of fixative, and dropped onto glass slides from a height of approximately 10 cm. Slides were air dried, stained in 2% Giemsa (Sigma), 67 mmol/l NaHPO₄, 67 mmol/l KH₂PO₄ and chromosomes examined using a Nikon light microscope (Nikon, Garden City, New York).

Statistical analysis

All data are expressed as mean $\pm$ SEM. Statistical significance of differences were determined by Student's t-test for independent samples (two-tailed).

Results

Impairment of humoral and cellular immunity in alloxan- and STZ-induced diabetic mice

The immune competence of alloxan- and STZ-induced diabetic mice was assessed at the humoral and cellular levels. Humoral immune function was determined by the ability of diabetic spleen cells to mount a primary antibody response to sheep red blood cells or a mitogenic response to concanavalin A upon challenge in vitro (Fig. 1). Cellular immune function was evaluated in vivo using the delayed-type hypersensitivity response to the hapten azobenzenearsonate (Fig. 1). In agreement with previous studies [10–12], severe depression of both arms of the immune system ($> 50\%$) was observed in alloxan- and STZ-induced diabetic mice relative to normal control mice. Equivalent levels of suppression were seen in alloxan- and STZ-diabetic mice. The level of insulin deficiency and metabolic control was also equivalent in STZ- and alloxan-diabetic mice. Pancreatic immunoreactive insulin levels were constant between days 4–10 after drug injection at 37 ± 4 µg/g for STZ-diabetic mice and 35 ± 5 µg/g for alloxan-diabetic mice; 33% and 31% respectively of normal levels. Mean glucose levels of all experimental mice were between 25–33 mmol/l. Mean weight loss of experimental mice relative to controls was 8% for both STZ and alloxan on day 5 after drug administration, and 17% (STZ) or 16% (alloxan) on day 10.

Fig. 1. Impaired humoral and cellular immune responses in diabetic mice. The immune responses of diabetic and normal mice was assessed by evaluating anti-sheep red blood cell plaque forming (▨), and concanavalin A mitogenic responses in vitro (▧), and delayed-type hypersensitivity responses to the hapten azobenzenearsonate in vivo (▩). The responses of alloxan and STZ-diabetic mice were assayed on day 5 following drug injection. Drugs were not added to in vitro incubations. Results are expressed as a percentage of the mean response of untreated (normal) mice $\pm$ SEM. Normal responses were: 2561 plaque forming cells/ 10^6 spleen cells for anti-sheep red blood cells assay; 98,451 cpm for concanavalin A stimulated proliferation; and 26×10^{-2} mm for delayed-type hypersensitivity measurements. $p < 0.001$, $n = 18$ for all tests of diabetic compared to normal mice.

Fig. 2A–D. Alloxan and STZ toxicity in vitro. The toxicity of alloxan (open symbols) or STZ (closed symbols) were measured in vitro on isolated **A** pancreatic islets, **B** splenocytes, **C** thymocytes, and **D** adherent splenic macrophages. Cell viability was measured after 2-day (circles) and 5-day (triangles) incubations with drug. Results presented are the means of experiments performed in quadruplicate. Mean $\pm$ SEM $< 3.0\%$ for all points, $n = 6$

Effect of alloxan and STZ on the viability and function of lymphoid cells

In light of previously reported direct suppressive effects of STZ on cellular immune responses, we undertook an analysis of the effects of alloxan and STZ, over a wide range of concentrations, on both cellular viability and relevant functional immune properties of lymphoid cells.

Table 1. Chromosomal abnormalities induced by alloxan or STZ

Treatment	% Cells aberrant	Chromatic exchanges	Chromatic breaks	Other aberrations	Mitotic ^a index
None	2	0	0	0	0.7
Alloxan exposure 2 days in vitro (mmol/l)					
1.0	3	0	3	0	0.6
2.0	3	0	2	2	1.4
4.0	2	0	2	0	0.7
5.0	3	0	3	0	0.9
7.0	6	0	9	2	1.1
STZ exposure 2 days in vitro (mmol/l)					
0.4	2	1	3	0	1.6
1.0	4	0	3	1	1.2
2.0	14	3	13	9	1.1
3.0	23	9	23	26	1.0
4.0	0 ^b	0 ^b	0 ^b	0 ^b	<0.2
Alloxan administration 2 days in vivo (n = 8)					
50 mg/kg	4	0	3	1	1.4
STZ administration 2 days in vivo (n = 8)					
150 mg/kg	10	2	8	6	1.5

1000 cells analyzed/group. ^aThe mitotic index was calculated from the number of mitotic figures/1000 cells counted. ^bViability <20%

Table 2. Distribution of splenic lymphocyte populations in normal versus alloxan-diabetic mice

Lymphocyte Class	Cells stained/140,000	% of total spleen cells	% of splenic T cells
B cells (n = 8)			
Normal	20,984 ± 348	52	-
Diabetic	21,072 ± 467	53	-
T cells (n = 8)			
Normal	10,032 ± 261	25	-
Diabetic	8,744 ± 297	22	-
Lyt 1 ⁺ T cells (n = 8)			
Normal	23,908 ± 621	-	60
Diabetic	22,536 ± 811	-	56
Lyt 2 ⁺ T cells (n = 8)			
Normal	6,740 ± 148	-	17
Diabetic	6,020 ± 156	-	15

Data on the staining of T cells and T-cell subsets have been corrected for staining detected in the absence of T-cell specific antibody markers

The effects of alloxan and STZ on the viability of pancreatic, splenic, thymic, and adherent macrophage populations are shown in Figure 2. Both drugs were toxic for purified pancreatic islet cells at >0.2 mmol/l in 5 day incubations (Fig. 2A). Significant toxicity was also seen after 2 days of incubation, with the toxicity of STZ being more pronounced than that of alloxan. The toxicity of alloxan and STZ toward spleen, thymus and macrophage cells showed a markedly different though consistent pattern (Fig. 2B–D). In each instance no alloxan toxicity was detected on days 2 or 5 at any concentration, while STZ toxicity was seen beginning at doses of 1–2 mmol/l.

Fig. 3. Effect of alloxan and STZ on the anti-sheep red blood cell plaque-forming response in vitro. Anti-sheep red blood cell plaque responses were conducted in vitro in a 5-day incubation protocol in the presence of either alloxan (open symbols) or STZ (closed symbols). Drugs were present for either the entire 5-day incubation (triangles) or were added for only the last 2 days of culture before assay (circles). The means of experiments performed in triplicate are presented. Mean $\pm$ SEM <8.0% for all points, $n = 6$

The effects of alloxan and STZ on the function of cells, which govern immunity, were assessed by monitoring exposure to a range of drug concentrations in vitro on: (1) the sheep red blood cell antibody response (Fig. 3); (2) the proliferative response of splenocytes to the T-cell mitogens concanavalin A and phytohaemagglutinin (Fig. 4A, B), and the B-cell mitogen lipopolysaccharide (Fig. 4C); (3) the synthesis of interleukin-2 by concanavalin A activated splenocytes (Fig. 5A); and (4) the synthesis of interleukin-1 by lipopolysaccharide stimulated splenic macrophages (Fig. 5B). In agreement with the preceding results, STZ routinely inhibited cellular functions at concentrations >0.4 mmol/l, while alloxan had no effect at concentrations <10 mmol/l.

Mechanism of STZ and alloxan toxicity

Despite their differences in structure, the mechanisms of alloxan and STZ toxicity on pancreatic islet cells are believed to be similarly mediated by oxidative DNA strand breakage [9]. The incidence of chromosomal abnormalities in splenocytes upon exposure to alloxan or STZ, in vitro or in vivo, is presented in Table 1. Only STZ induced chromosomal aberrations on in vitro exposure; these were seen at concentrations >2 mmol/l. Spleen cells isolated from mice 2 days following injection with diabetogenic doses of alloxan (1.1 mmol/l) or STZ (1.8 mmol/l) were analyzed in a similar manner. Spleen cells from alloxan-induced diabetic mice showed no signs of abnormalities while those from STZ-induced diabetic mice exhibited low but significant levels of chromosomal abnormalities ($p < 0.005$, $n = 8$).

Fig. 4. Effect of alloxan and STZ on the mitogenic response of splenocytes in vitro. Proliferative responses of splenocytes to the mitogens **A** phytohemagglutinin, **B** concanavalin A and **C** lipopolysaccharide were conducted in vitro in the presence of either alloxan (open symbols) or STZ (closed symbols). Reactions were pulsed with ^3H -thymidine for the final 8 h of 48 h incubations. The mean ^3H -thymidine incorporation of experiments conducted in quadruplicate are presented. Mean $\pm$ SEM $< 4.0\%$ for all points, $n = 6$

Immune response of insulin-treated diabetic mice

Final assessment of the usefulness of a drug-based model for studying the immune competence of diabetic mice was accomplished by comparing the responsiveness of normal, alloxan- and STZ-diabetic mice to that of insulin-treated alloxan- and STZ-diabetic mice. The degree of metabolic control achieved in mice treated for 4 days with insulin was determined by plasma glucose levels, weight measurements, and glucose tolerance tests. Insulin-treated alloxan- and STZ-diabetic mice exhibited nearly normal levels of control. Plasma glucose levels were reduced on insulin treatment to within 10% of normal: 135 ± 12 mmol/l for alloxan and 131 ± 16 mmol/l for STZ. Alloxan-treated mice experienced a 4% weight gain and STZ-treated mice a 3% weight gain over the first 4 days. Treated alloxan and STZ-diabetic

Fig. 5. Effect of alloxan and STZ on the production of interleukin-1 and interleukin-2 in vitro. **A** Interleukin-2 synthesis by concanavalin A stimulated splenocytes. **B** Interleukin-1 synthesis by lipopolysaccharide stimulated macrophages. Incubations were conducted for 48 h in the presence of either alloxan (open symbols) or STZ (closed symbols). Results are presented as the mean ^3H -thymidine incorporation of appropriate indicator cells and were performed in triplicate. Mean $\pm$ SEM $< 3.0\%$ for **A** and $< 4.0\%$ for **B**, $n = 6$

mice had equivalent yet slightly elevated glucose tolerance responses. The responses of normal mice were 146 ± 11 mmol/l, while insulin-treated alloxan-diabetic mice were 185 ± 19 mmol/l and insulin-treated STZ-diabetic mice were 172 ± 12 mmol/l ($n = 18$ for all determinations).

Immune competence was assayed, as described in Figure 1, for in vitro concanavalin A mitogenesis and anti-sheep red blood cell responses, and in vivo by delayed-type hypersensitivity responses. The results indicate that insulin-treated alloxan-diabetic mice respond at normal levels (Fig. 6), thus reinforcing the conclusion drawn previously that a diabetogenic dose of alloxan (50 mg/kg) does not itself cause immune dysfunction. In contrast, the response of insulin-treated STZ-diabetic mice remained slightly impaired relative to normal levels. Although insulin management reversed the STZ-diabetic effect by 70 to 80% of normal and returned blood glucose levels to the normal range, the lack of a complete recovery indicates that diabetogenic doses of STZ (150 mg/kg) result in direct, irreversible damage to components of the immune system.

Distribution of splenic lymphocyte populations in alloxan-diabetic mice

In light of recent reports of severe T-cell lymphocytopenia in the BB diabetic rat [29] and a reduced splenic weight in alloxan-diabetic mice [11, 14, 28], we also in-

Fig. 6. Humoral and cellular immune responses of insulin-treated diabetic mice. Anti-sheep red blood cells plaque forming responses (■), concanavalin A proliferation (▨), and delayed-type hypersensitivity responses (▩) were conducted on alloxan-diabetic and insulin-treated alloxan-diabetic mice, and STZ-diabetic and insulin-treated STZ-diabetic mice as described in Figure 1. $n=18$ for all groups. *, $p<0.001$; **, $p<0.002$; +, $p<0.05$; ++, $p<0.02$

investigated the possibility that alloxan-diabetic mice have an altered ratio of B- and T-cell subsets. Fluorescence analysis of spleens from 5-day alloxan-diabetic mice displayed a normal pattern of B- and T-cell subset proportions (Table 2). Histological analysis of alloxan-diabetic spleen and thymus (not shown) demonstrated a normal morphology on day 2 and arteriolar thickening associated with establishment of diabetes on day 5. No necrotic lesions were detected on days 2 or 5.

Discussion

The results of this study indicate that the use of alloxan to induce the diabetic syndrome provides a useful model to study the immune system in diabetic mice. Studies in vivo and in vitro demonstrated that even at greater than ten times the diabetogenic dose (1.1 mmol/l) alloxan was neither directly toxic for lymphoid cells nor did it irreversibly impair cellular functions critical to generation of the immune response. Most importantly, normal immune responses were restored in alloxan-diabetic mice which were managed by thrice daily injections of insulin. Results supportive of this hypothesis have also been reported by Ptak et al. [11].

In contrast, the diabetogenic drug STZ displayed significant lymphoid cell toxicity in vitro, and impaired lymphoid cell mediated functions in vitro and in vivo at doses equivalent to or less than those required for the establishment of diabetes (1.8 mmol/l). Management of STZ-diabetic mice with insulin failed to completely restore immune impairment. Results of a similar nature have been reported by Nichols et al. [14] for STZ depression of blastogenic and cytolytic responsiveness.

Therefore, while the in vitro toxicity of STZ for pancreatic islet B cells is more pronounced than on lymphoid cells, the window of cross-sensitivity in vivo overlaps to an extent which results in significant non-specific lymphoid toxicity. Our karyotype analyses suggest that the primary mechanism of STZ lymphoid toxicity is via oxidative driven mutagenesis in rapidly dividing

cells. These results stress caution in the use of STZ for the study of other pathological phenomena associated with diabetes and in the treatment of pancreatic islet cell carcinomas.

The window of alloxan cross-sensitivity as measured in vitro is considerably larger than with STZ. Coupled with the three-fold lower diabetogenic dose of alloxan relative to STZ, this window is maintained in vivo and allows for B-islet cell destruction in the absence of significant lymphoid toxicity. One explanation for why insulin restores immune competence in alloxan-induced diabetes may be simply that of improved glucose transport in lymphocytes which must proliferate and metabolize rapidly following activation with antigen.

Acknowledgements. Dr. Gaulton is the recipient of a National Institutes of Health postdoctoral fellowship No. 5-F32-CA-06948-02. Supported by grant AI 20842 from the National Institutes of Health.

References

- Johnson JE III (1983) Infection and diabetes. In: Ellenberg M, Rifkin H (eds) Diabetes mellitus: Theory and practice. McGraw-Hill, New York, pp 734-7452
- Louria DB (1967) Deep-seated mycotic infections, allergy to fungi and mycotoxins. *N Engl J Med* 277: 1065-1071
- MacCuish AC, Jordan J, Campbell CJ, Duncan LJP, Irvine WJ (1974) Cell-mediated immunity to human pancreas in diabetes mellitus. *Diabetes* 23: 693-697
- Buse JB, Rowley RF, Eisenbarth GS (1982) Disordered cellular immunity in type I diabetes of man and the BB rat. *Surv Immunol Res* 1: 339-351
- Coleman DL (1979) Diabetes mellitus in rodents. In: Andrews EJ, Ward BC, Altman NH (eds) Spontaneous animal models of human disease, vol 1. Academic Press, New York, pp 126-131
- Rerup CC (1970) Drugs producing diabetes through damage of the insulin secreting cells. *Pharm Rev* 22: 485-518
- Weiss RB (1982) Streptozotocin: a review of its pharmacology, efficacy, and toxicity. *Cancer Treatment Reports* 66: 427-436
- Malaisse-Lagae F, Sener A, Malaisse WJ (1981) Biochemical basis of a species difference in sensitivity to alloxan. *FEBS Lett* 133: 181-182
- Yamamoto H, Uchigata Y, Okamoto H (1981) Streptozotocin and

- alloxan induce DNA strand breaks and poly(ADP-ribose) synthetase in pancreatic islets. *Nature* 294: 284–286
10. Ptak W, Hanczakowska M, Rozycka R, Rozycka D (1977) Impaired antibody responses in alloxan diabetic mice. *Clin Exp Immunol* 29: 140–146
 11. Ptak W, Czarnik Z, Hanczakowska M (1975) Contact sensitivity in alloxan-diabetic mice. *Clin Exp Immunol* 19: 319–325
 12. Mahmoud AAF, Rodman HM, Mandel MA, Warren KS (1976) Induced and spontaneous diabetes mellitus and suppression of cell-mediated immunologic responses. *J Clin Invest* 57: 362–367
 13. Nevalainen DE, Hoftiezer U (1977) The response of C3H mice to streptozotocin. I. Thymic depression and leukocyte toxicity. *Am J Med Technol* 43: 417–418
 14. Nichols WK, Spellman JB, Vann LL, Daynes RA (1979) Immune responses of diabetic animals: direct immunosuppressant effects of streptozotocin in mice. *Diabetologica* 16: 51–57
 15. Hyvarinen A, Nikkila E (1962) Specific determination of blood glucose with 0-toluidine. *Clin Chim Acta* 1: 140–143
 16. Yoon JW, Onodera T, Nokin AL (1978) Virus-induced diabetes mellitus. XV. Beta cell damage and insulin-dependent hyperglycemia in mice infected with Coxsackie B4. *J Exp Med* 148: 1068–1080
 17. Onodera T, Toniolo A, Ray UR, Jenson AB, Knazek RA, Notkins AL (1981) Virus-induced diabetes mellitus XX. Polyendocrinopathy and autoimmunity. *J Exp Med* 153: 1457–1473
 18. Bernstein SE (1966) Physiological characteristics. In: Green EL (ed) *Biology of the laboratory mouse*, 2nd edn. McGraw-Hill, New York, pp 337–350
 19. Lee JM, DeWitt S Jr (1952) Studies in alloxan metabolism. *J Biol Chem* 197: 205–214
 20. Lacy PE, Kostianovsky M (1967) Method for the isolation of intact islets of langerhans from the rat pancreas. *Diabetes* 16: 35–39
 21. Cowing C, Schwartz BD, Dickler HB (1978) Macrophage Ia antigens. I. Macrophage populations differ in their expression of Ia antigens. *J Immunol* 120: 378–384
 22. Grankvist K, Lernmark A, Taljedal I-B (1977) Microscope photometric analyses of trypan blue uptake by pancreatic islet cells in suspension. *Biochem J* 162: 19–24
 23. Cunningham AJ (1965) A method of increased sensitivity for detecting single antibody-forming cells. *Nature* 207: 1106–1107
 24. Eardley DD, Murphy DB, Kemp JD, Shen FW, Cantor H, Gershon RK (1980) Ly-1 inducer and ly-1,2 acceptor T cells in the feedback suppressor circuit bear an I-J subregion determinant. *Immunogenetics* 11: 549–553
 25. Gillis S, Ferm MM, Ou W, Smith KA (1978) T cell growth factor: parameters of production and a quantitative microassay for activity. *J Immunol* 120: 2027–2032
 26. Mizel SB, Oppenheim JJ, Rosenstreich DL (1978) Characterization of lymphocyte-activating factor (LAF) produced by the macrophage cell line P388D1. 1. Enhancement of LAF production by activated T lymphocytes. *J Immunol* 120: 1497–1503
 27. Bach BA, Sherman L, Benacerraf B, Greene MI (1978) Mechanisms of regulation of cell-mediated immunity. II. Induction and suppression of delayed-type hypersensitivity to azobenzene-arsenate-coupled syngeneic cells. *J Immunol* 121: 1460–1468
 28. Nichols WK, Spellmann JB, Daynes RA (1978) Immune responses of diabetic animals: comparison of genetically obese and streptozotocin-diabetic mice. *Diabetologica* 14: 343–349
 29. Jackson R, Rassi N, Crump T, Haynes B, Eisenbarth GS (1981) The BB Diabetic rat: profound T-cell lymphocytopenia. *Diabetes* 30: 887–889

Received: 1 October 1984
and in revised form: 21 June 1985

Dr. Glen Gaulton
Department of Pathology
University of Pennsylvania
Medical School
3400 Spruce Street
Philadelphia, PA 19104
USA