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Studies of streptozotocin-induced insulitis and diabetes

(pancreatic beta cells/islets of Langerhans/alloxan diabetes/type C virus induction/cell-mediated reaction)

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ABSTRACT **Multiple small injections of streptozotocin produce a delayed, progressive increase in plasma glucose in mice within 5–6 days after the injections, in association with pronounced insulitis and induction of type C viruses within beta cells. Multiple subdiabetogenic doses of streptozotocin in rats and multiple injections of another beta cell toxin, alloxan, in mice did not induce insulitis although hyperglycemia followed the injection of larger quantities of both agents. In mice, the prior injection of 3-*O*-methyl-D-glucose (3-OMG) or nicotinamide attenuated the diabetic syndrome produced by streptozotocin; however, 3-OMG was more protective. Rabbit anti-mouse lymphocyte serum, alone, provided partial protection but, when given together with either 3-OMG or nicotinamide, effectively prevented the streptozotocin-induced diabetic syndrome. Cessation of these preventive treatments was followed by the appearance of insulitis and diabetes. These findings suggest that multiple injections of streptozotocin induce, in susceptible hosts, the triad of direct beta cell cytotoxicity, virus induction within beta cells, and cell-mediated autoimmune reaction. These factors, acting separately or in concert, appear to induce a destructive insulitis and severe diabetes. The relative importance of each component and the factors governing host susceptibility remain to be clarified.**

Streptozotocin [SZ; 2-deoxy-2(3-methyl-3-nitrosoureido)-D-glucopyranose] is a broad-spectrum antibiotic with oncolytic, oncogenic, and diabetogenic properties (1–4). The diabetogenic action is mediated by selective destruction of pancreatic beta cells and has been widely utilized as a method for inducing diabetes mellitus in experimental animals and for treatment of malignant beta cell tumors and other neoplasms in humans. To produce diabetes, SZ is conventionally administered as a single injection. SZ is cleared from the bloodstream rapidly (serum half-life, 15 min) (5); beta cell necrosis can be detected by electron microscopy within hours after SZ injection (6–8). Elevated blood glucose levels are demonstrable within 1–2 days, and dissolution and phagocytosis of necrotic cells are observed histologically after 3 days (3, 6). In rats and mice, the administration of a single subdiabetogenic dose produces only mild histologic alterations without evidence of significant hyperglycemia when compared with buffer-injected control animals (9, 10).

We have recently reported (10) that the administration of multiple (five) subdiabetogenic doses of SZ to Charles River Laboratory (CD-1) mice, either intravenously or intraperitoneally, produced a delayed but progressive increase in blood glucose with levels of 350–450 mg/100 ml within 5–6 days after the last injection. Morphologically, the pancreatic islets revealed pronounced insulitis with infiltrating lymphocytes and macrophages, architectural distortion, and beta cell necrosis. There was a subsequent decrease in inflammation within the remaining islets which were small and composed almost exclusively of non-beta cells. Blood glucose values remained in the diabetic range and correlated well with the pathologic changes within the islets. Noteworthy also was the presence of numerous type C viruses within the surviving beta cells of animals studied within 6 days of the last SZ injection. The delayed development of the insulitis and the nature of the inflammatory infiltrate appeared to suggest a cell-mediated immune reaction. The role of the increased number of type C viruses within the beta cell remains to be clarified.

In this paper we report additional studies of the streptozotocin-induced insulitis model of diabetes. Various substances that alter or attenuate the insulitis were studied, including 3-*O*-methyl-D-glucose (3-OMG) and nicotinamide which have been shown to protect against the single-injection method of inducing diabetes (9). Antilymphocyte serum (ALS), which has been used to inhibit cell-mediated graft rejection (11–13), was used to determine indirectly whether the SZ injections trigger a cell-mediated autoimmune reaction directed against the pancreatic beta cells. Furthermore, the specificity of the response to SZ was evaluated by comparison with another beta-cytotoxic diabetogenic agent, alloxan. Finally, experiments evaluating the response of rats to multiple injections of SZ were also carried out to determine if insulitis and hyperglycemia could be induced in another species.

MATERIALS AND METHODS

Animals. Male rats (Charles River strain of Sprague-Dawley) weighing 190–200 g and male mice (Charles River CD-1 strain) weighing 35–40 g were obtained from Charles River Breeding Company, Wilmington, MA. The rats were given free access to water and Purina Rat Chow; the mice were given Old Guilford Mouse Pellets (96W).

Toxic Compounds. Streptozotocin (U9889, lots 11837-GGS-22B and 1676-RCH-112) was kindly supplied by W. E. Dulin of the Upjohn Co., Kalamazoo, MI. The crystalline SZ was assayed to be approximately 90% α anomer based on optical rotation data. SZ was dissolved in a citrate buffer, pH 4.5, and injected intravenously or intraperitoneally within 15 min of dissolution. Alloxan monohydrate (Eastman Co., Rochester, NY) was dissolved in 0.9% NaCl just prior to use and was injected intravenously in a volume of 0.1 ml. An additional 0.2 ml of 0.9% NaCl was given to flush the solution through the tubing.

Histology and Plasma Glucose Determinations. For light microscopy, the pancreata were fixed in Bouin's solution, and sections were stained with hematoxlin and eosin and aldehyde fuchsin. For electron microscopy, tissues were fixed in a solution

Abbreviations: SZ, 2-deoxy-2(3-methyl-3-nitrosoureido)-D-glucopyranose; ALS, antilymphocyte serum; 3-OMG, 3-O-methyl-D-glucose.

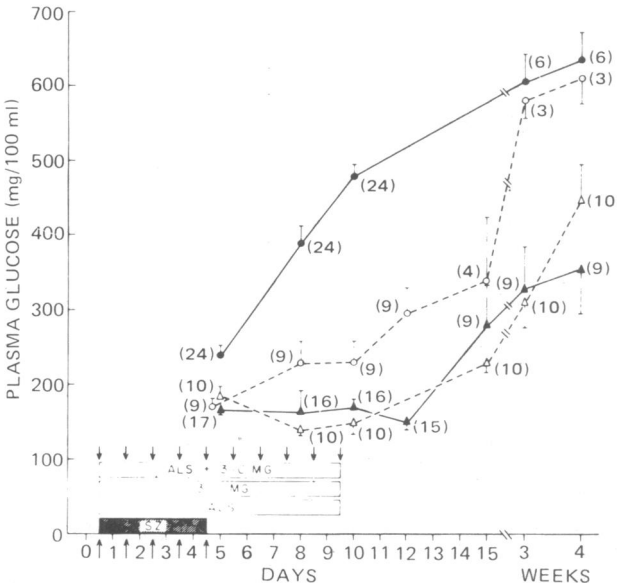

FIG. 1. Plasma glucose (mean ± SEM) levels in CD-1 mice after: 5 daily intraperitoneal injections of SZ (40 mg/kg) (●); 10 daily intraperitoneal injections of ALS (0.5 ml) plus SZ (○); 10 intraperitoneal injections of 3-OMG (0.22 mmol per mouse) plus SZ (▲); or 10 intraperitoneal injections of 3-OMG plus ALS plus SZ (△). Numbers in parentheses are numbers of determinations.

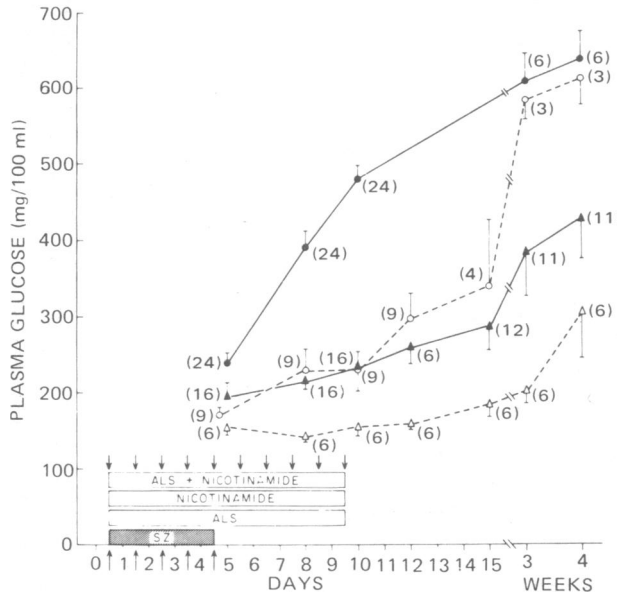

FIG. 2. Plasma glucose levels (mean ± SEM) in CD-1 mice after: 5 intraperitoneal injections of SZ (40 mg/kg) (●); 10 intraperitoneal injections of ALS (0.5 ml) plus SZ (○); 10 intraperitoneal injections of nicotinamide (0.22 mmol per mouse) plus SZ (▲); or 10 intraperitoneal injections of ALS plus nicotinamide plus SZ (△). Numbers in parentheses are numbers of determinations.

of paraformaldehyde and glutaraldehyde as reported (14). Blood samples for glucose determination were collected from fed animals into heparin-treated pipettes and assayed as described previously (10).

Other Substances. Nicotinamide and 3-OMG were obtained from the Sigma Chemical Co., St. Louis, MO. The 3-OMG was dissolved in 0.9% NaCl at least 2 hr prior to injection to ensure mutarotation of the anomers to equilibrium. The solution was injected in a volume of 0.1 ml (0.22 mmol per mouse). Nicotinamide was prepared in a similar manner, and 0.22 mmol per mouse was injected intraperitoneally in a volume of 0.1 ml.

Rabbit anti-mouse lymphocyte serum (ALS) and normal (nonimmune) rabbit serum were obtained from Microbiological Associates, Bethesda, MD. The ALS preparations were tested by hemagglutination, cytotoxicity, and skin allograft prolongation:‡ lot 15172, cytotoxic at 1:3200, caused hemagglutination at 1:128, and resulted in skin graft prolongation of 30.3 ± 1.8 days; lot 15096, cytotoxic at 1:6400, caused hemagglutination at 1:64, and resulted in skin graft prolongation of 30.1 ± 3.3 days. ALS and normal rabbit serum were injected intraperitoneally, each in volumes of 0.5 ml.

The sequence of injections was as follows: first, ALS, normal rabbit serum, nicotinamide, or 3-OMG; and then SZ. A time lapse of 5–15 sec occurred between intraperitoneal injections.

RESULTS

Attenuation of SZ-induced insulitis by 3-OMG, nicotinamide, and ALS

The prior injection of 3-OMG or nicotinamide has been found to protect against SZ-induced beta cell necrosis in rats (9) and mice (CD-1 and DBA/2J strains; unpublished data), when SZ is administered as a single diabetogenic dose. These compounds therefore were given prior to each of the multiple SZ injections. In each instance, the daily administration of 3-OMG, nicotinamide, or ALS was continued for 5 days after completion of the SZ injections.

Fig. 1 illustrates the results of glucose determinations over a 4-week period during which SZ was given for 5 days alone or in combination with either 3-OMG (0.22 mmol per mouse) or ALS (0.5 ml per mouse), or both 3-OMG and ALS. In agreement with our previous findings (10), glucose levels in SZ-injected mice were elevated on the fifth day of injections and continued to increase progressively in the subsequent 3-week period. The animals receiving the combination of 3-OMG and SZ evidenced no increase in glucose for the initial 12 days; however, by the 15th day, 5 days after the cessation of 3-OMG injections, glucose elevations were observed and persisted for the following 2 weeks. It is noteworthy that plasma glucose levels in this group were significantly lower than in those mice receiving SZ alone. Among the animals sacrificed on the 12th day of the experiment, histologic examination revealed only an occasional round cell infiltrating or surrounding the otherwise normal-appearing pancreatic islets.

In Fig. 2, the plasma glucose levels are shown after injection of SZ alone or with nicotinamide (0.22 mmol per mouse) prior to the SZ. Glucose levels in the nicotinamide-treated animals were slightly elevated by the 5th day (mean ± SEM, 196 ± 19 mg/100 ml, n = 16); by the 12th day, mean plasma glucose was 261 ± 23 mg/100 ml (n = 16). Histologic examination on the 12th day revealed a greater number of inflammatory cells permeating the islets compared to the 3-OMG and SZ group but markedly decreased compared to the group receiving SZ alone. By the 4th week, plasma glucose was 428 ± 53 mg/100 ml (n = 11). Thus, at an equimolar concentration, 3-OMG appeared to provide greater protection than did nicotinamide. The protection was incomplete, however, because the plasma glucose levels were elevated by the 4th week.

To determine if the infiltration of lymphocytes within the pancreatic islets represents a cell-mediated immune reaction against the beta cells, the effect of rabbit ALS on SZ-induced insulitis was studied. ALS (0.5 ml intraperitoneally) given alone

‡ Determinations performed by Microbiological Associates.

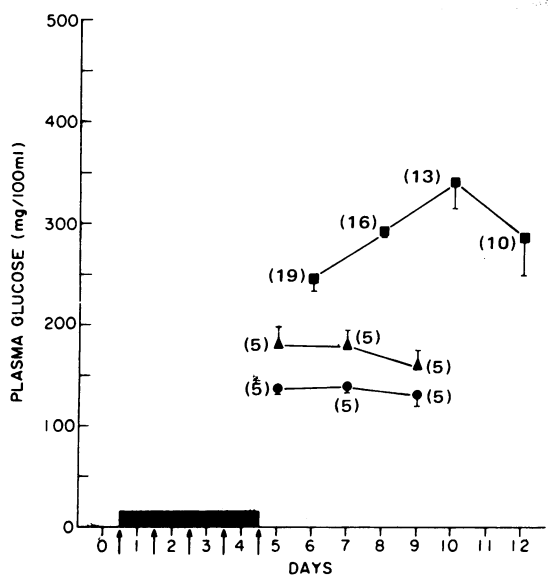

FIG. 3. Plasma glucose levels (mean ± SEM) in Charles River rats after five daily intravenous injections of varying SZ doses: ■, 15 mg/kg; ▲, 10 mg/kg; ●, 5 mg/kg. Numbers in parentheses are numbers of rats in each group. Mean plasma glucose of normal fed rats is 129 ± 4 mg/100 ml (n = 12).

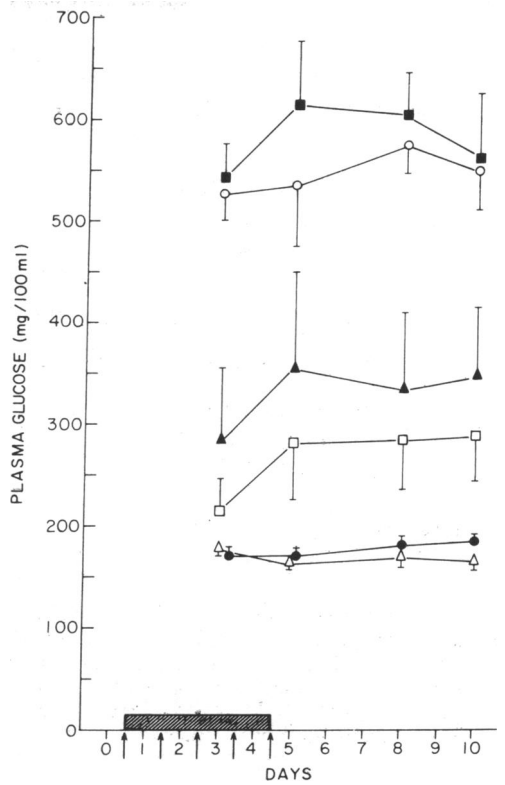

FIG. 4. Plasma glucose levels (mean ± SEM) in Charles River CD-1 mice (n = 6) after five daily intravenous injections of varying alloxan doses: ■, 50 mg/kg; O, 60 mg/kg; ▲, 40 mg/kg; □, 30 mg/kg; ●, 20 mg/kg; △, 10 mg/kg.

for 11 consecutive days caused a slight nonspecific decrease in the plasma glucose in otherwise untreated mice, perhaps due to a decrease in food intake. Figs. 1 and 2 illustrate that, when both ALS and SZ were administered for 5 days and ALS injections were continued for an additional 6 days, the elevation in glucose was 50% less than that induced by SZ alone. Histologic examination of ALS-treated animals sacrificed on day 11 revealed a marked decrease of the inflammatory cell infiltrate within the islets. However, beta cell injury and partial degranulation as well as architectural disarray were noted. Animals that were not sacrificed revealed, in the following weeks, a progressive increase in plasma glucose levels, with values comparable to those achieved in mice receiving multidose SZ injections alone. In separate experiments (data not shown), daily injections of normal rabbit serum prior to SZ did not prevent either the glucose elevations or the appearance of insulitis.

Also shown in Fig. 1 are glucose levels in mice receiving the combination of 3-OMG, ALS, and SZ. By the 15th day, glucose levels were elevated and this progressive rise continued to frankly diabetic levels in the following 2 weeks. Animals sacrificed on the 12th day of the experiment revealed almost complete absence of insulitis. Subtle islet cell injury, similar to that in 3-OMG- and SZ-injected mice, was present. Thus, the combination of the 3-OMG and ALS provided a significant degree of protection, but only during the period of injections. The combination of nicotinamide and ALS (Fig. 2) provided greater protection during the course of injections. However, in the following 2 weeks, plasma glucose concentrations rose to diabetic levels, a finding similar to that observed in animals receiving ALS + 3-OMG + SZ or 3-OMG + SZ.

SZ toxicity in rats

Previous experiments in the rat with graded doses of SZ revealed that a single injection of 60 mg/kg induced diabetes within 24–48 hr, with blood glucose levels of 440 ± 10 mg/100 ml (n = 12) (9). In the present studies, SZ was administered intravenously in five daily divided doses of 5, 10, or 15 mg/kg per day.

Fig. 3 shows the plasma glucose levels at various time intervals after the three groups of five SZ injections. At a dose of 5 mg/kg per day, glucose levels were not significantly elevated. A slight increase was observed in animals receiving 10 mg/kg per day, but plasma glucose levels returned to normal by the 11th day. At 15 mg/kg per day, however, glucose was elevated to 240 mg/100 ml by the 5th day and reached an apogee by the 12th day. Histologic examination of the islets revealed no evidence of significant inflammation at any of the three SZ dosages. However, beta cell degranulation and architectural disarray were observed in animals receiving the higher doses of SZ and could be correlated with the glucose elevations. In animals receiving SZ at 10 or 15 mg/kg per day (total doses, 50 and 75 mg/kg, respectively), plasma glucose elevations were similar to those observed 24–48 hr after the administration of a single injection of SZ at approximately 30–35 mg/kg.

Alloxan toxicity studies in mice

To determine if the insulitis induced by multiple SZ injections in CD-1 mice was due to nonspecific beta cell injury, alloxan, another beta cell toxin, was studied. A dose-response curve was first determined with glucose levels at 48 hr after graded doses of alloxan administered as a single intravenous injection in animals fasted overnight. At dosages of 10–20 mg/kg, plasma glucose levels were not significantly elevated; at 50 mg/kg and greater, glucose levels reached the diabetic range (approximately 500 mg/100 ml) and persisted at this level for the remaining 10 days of the experiment. Fig. 4 illustrates glucose levels in mice after intravenous injection of alloxan at 10–60 mg/kg per day for 5 days. At 10 and 20 mg/kg per day, there was no elevation of glucose when compared with normal, fed, untreated mice (160 ± 5 mg/100 ml; n = 34). Mice receiving 30 and 40 mg/kg per day evidenced maximal glucose elevation

on the fifth day of injections and stabilization at this level for the duration of the experiment. At 50 and 60 mg/kg per day, glucose levels above 500 mg/100 ml were observed by the 5th (last) day of injection and remained at this level until the time of sacrifice on day 10.

Histologically, the pancreatic islets of mice receiving the smaller alloxan doses (10–40 mg/kg) revealed only mild beta cell degranulation and subtle architectural disarray. With higher doses (50–60 mg/kg), more pronounced architectural changes were observed. Islet size was decreased and evidence of extensive beta cell degranulation and focal necrosis was observed; noteworthy, however, was the absence of any significant inflammatory cell infiltrate at any of the dosage levels. The lack of an additive toxic effect of alloxan was observed because at 10 or 20 mg/kg daily for 5 days the total alloxan dose is 50 or 100 mg/kg, which is sufficient to produce diabetes if administered as a single injection.

DISCUSSION

Insulitis, the lymphocytic infiltration of the islets of Langerhans, has been observed in children dying shortly after the diagnosis of juvenile-onset diabetes (15). Although the etiology of this pathologic finding is unknown, there is now evidence that this lesion can be produced in animals by various procedures. For example, the chronic injection of heterologous or homologous insulin has been shown to produce inflammatory lesions of the islets of Langerhans (16–19). In other experiments, the administration of antiserum to insulin has been found to produce a diffuse inflammatory process in the pancreas including the islets of Langerhans (20). The injection of mice with lyophilized isolated islets suspended in Freund's adjuvant has also been found to produce lymphocytic infiltration and beta cell degeneration (21). One of the major criticisms of the experimental production of insulitis by these methods has been the absence of severe or sustained carbohydrate intolerance in the animals with induced islet lesions. Another model of experimental diabetes that has been reported in recent years appears to provide support for the viral etiology of the juvenile diabetes syndrome. The injection of a specific virus strain (M variant of the encephalomyocarditis virus) into certain strains of adult mice produces an acute diabetic syndrome in approximately 40% of the animals, with histologic evidence of severe insulitis and beta cell necrosis (22, 23).

In the present study, multiple injections of subdiabetogenic quantities of SZ in CD-1 mice produced mild hyperglycemia during the initial 5–6 days of the experiment, with a complete diabetic syndrome observed by the 8th–11th day. The consistency of induction of pronounced hyperglycemia allows this model to be used without the necessity of glucose tolerance testing to reveal the presence of marginal glucose intolerance. Histologically, insulitis, first noted several days after the completion of injections, is most intense after 7–10 days and gradually decreases in magnitude with only mild residual inflammation present 3–4 weeks later. Islet architectural distortion and beta cell necrosis mirror the degree of insulitis; residual beta cells are degranulated and gradually diminish in number, and the remaining islets are small and composed almost exclusively of non-beta cells (10). These end-stage pathologic findings are similar to those observed 1 week after administration of a single diabetogenic dose of SZ or alloxan.

The relationship between the inflammatory response and the multiple injections of SZ remains to be elucidated. However, the specificity of the SZ-induced insulitis is supported by the failure to produce these lesions with alloxan. Multiple injections of alloxan did produce beta cell necrosis as well as a diabetic state which was correlated with the dosage of alloxan administered. Thus, the possibility that repeated, mild, nonspecific beta cell injury might be responsible for the induction of insulitis is not supported by the alloxan data. Although both alloxan and SZ produce diabetes, the two compounds appear to act by different mechanisms (24). SZ, which produces destruction of the beta cell with a single injection, is also capable of inducing insulitis when multiple subdiabetogenic injections are given. With alloxan, only the former technique is effective. The differential effect of various compounds in protecting the animals from the toxic effects of SZ and alloxan also suggests that their mechanisms of action are different (9, 24–26). The lack of an additive effect of alloxan and the absence of insulitis after multiple subdiabetogenic injections suggest that reparative processes can overcome the low-grade injury that results from small doses of alloxan without the initiation of an autoimmune reaction. Only when multiple larger doses of alloxan were injected was there sufficient beta cell destruction to produce diabetes. Again, insulitis was absent and the effects were essentially identical with those after a single diabetogenic injection.

The specificity of the SZ-induced insulitis was suggested also by the lack of response when rats were used. Although the technique used to calculate the dosage necessary to induce insulitis was similar to that used for the mice, there was no evidence of insulitis in the injected rats. This was in spite of the fact that sufficient quantities of SZ were used to induce diabetes and architectural changes within the islets.

The mechanism of protection by nicotinamide and 3-OMG in rats necessitates the injection of 3-OMG within minutes prior to the administration of SZ, whereas nicotinamide is capable of protecting the islets when given 2 hr after SZ injection (9, 27–29). The ability of 3-OMG and nicotinamide to provide considerable protection against SZ-induced insulitis in mice suggests that the direct beta cytotoxic action of SZ is an important prerequisite for the induction of insulitis. The fact that the duration of 3-OMG and nicotinamide protection does not exceed 2 weeks suggests that this protection is not complete. This conclusion is supported by histologic evidence of low-grade insulitis in mice sacrificed during the 2-week period of "protection" (as determined by blood glucose levels). The combination of ALS and SZ greatly inhibited the magnitude of the insulitis; however, diabetes was not prevented, presumably as a result of direct (not immune-mediated) SZ toxicity. This impression is supported by the greater protection afforded by the combined administration of 3-OMG, ALS, and SZ, wherein 3-OMG is assumed to have diminished the magnitude of direct SZ beta cell toxicity and ALS to have protected against the lymphocyte-mediated injury. However, studies with various treatment regimens of ALS, along with varying SZ doses, will be needed to establish more conclusively the role of the cell-mediated reaction.

Thus, there appear to be two or three factors that produce the insulitis. The first is a direct toxic action of SZ that is additive in the mouse. The direct beta cell toxicity of SZ does not appear to be shared by alloxan in mice or to be produced by SZ in rats. The second factor is a cell-mediated autoimmune reaction directed against the pancreatic beta cells. Almost complete inhibition of the lymphocytic infiltrate was accomplished by the administration of ALS, with partial amelioration of hyperglycemia. When both 3-OMG and ALS were given, direct SZ (non-immune) injury and lymphocyte-mediated injury were presumably prevented. Insulitis and hyperglycemia were not observed in these mice during the period of 3-OMG and ALS injections. When these agents were discontinued, plasma glucose elevation occurred within 2–3 weeks.

A third factor may be the role of type C virus in the etiology of insulitis. We have already suggested (10) that this RNA virus, considered to be part of the murine genome and known not to be directly cytotoxic, might act by inducing immunogenic proteins within the beta cells that are responsible for the initiation of an autoimmune reaction. To speculate further, the diabetic syndrome may be related to the virus if the function of the beta cell has been altered, by the action of SZ, from that of an insulin-producing cell to a "cell primarily synthesizing virus." This may be similar to the phage-induction phenomenon in bacteria.

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