JCI The Journal of Clinical Investigation

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J Clin Invest. 1976;57(2):362-367. https://doi.org/10.1172/JCI108287.

Research Article

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Induced and Spontaneous Diabetes Mellitus and Suppression of Cell-Mediated Immunologic Responses

GRANULOMA FORMATION, DELAYED DERMAL REACTIVITY, AND ALLOGRAFT REJECTION

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ABSTRACT These investigations delineate the recently described suppression of a form of cellular hypersensitivity in mice with streptozotocin-induced diabetes mellitus using a variety of cell-mediated immunologic responses in animals with several different forms of diabetes. Streptozotocin- and alloxan-induced diabetic mice and db/db genetically determined diabetic mice showed reductions in the areas of inflammation around *Schistosoma mansoni* eggs injected into the pulmonary vasculature of 68, 70, 77%, respectively. In contrast, streptozotocin-induced diabetes had no effect on the nonimmunologic foreign body granuloma around divinyl benzene copolymer beads injected into the pulmonary arterioles. Animals protected from diabetes by treatment with nicotinamide before streptozotocin administration did not develop hyperglycemia and had normal areas of immunologic granuloma formation around schistosome eggs. Treatment with insulin reversed the suppression of schistosome egg granuloma formation in both streptozotocin- and alloxan-diabetic animals. Two additional in vivo parameters of cellular immunologic reactivity were examined in streptozotocin-induced diabetes: delayed footpad swelling was essentially eliminated; skin graft survival across the *H-2* area was significantly prolonged from 10.2 days in the controls to 14.4 days in moderately diabetic A/J mice. These observations suggest that diabetes mellitus is associated with suppression of cell-mediated reactions in vivo and that the defect is reversible with insulin treatment.

An abstract of this work was published in *Clin. Res.* 23: 422A, 1975.

Received for publication 11 July 1975 and in revised form 13 October 1975.

INTRODUCTION

Infectious diseases, particularly tuberculosis, were a major cause of death among patients with diabetes mellitus before the advent of insulin therapy (1). Thus, abnormalities in the natural defenses against infection in diabetics have long been suspected and sought (2, 3). In 1964 Bybee and Rogers (4) showed that phagocytosis was impaired in ketotic diabetics. Later, Bagdade et al. (5) made similar observations in patients with hyperglycemia but without ketoacidosis and showed that the defect in phagocytosis disappeared with insulin therapy. They also reported that both diabetic serum and glucose added to normal serum inhibited phagocytosis by normal granulocytes (6). In addition, Mowat and Baum (7) have reported inhibition of chemotaxis of polymorphonuclear leukocytes in patients with diabetes.

With respect to immunologic mechanisms, the few reports of humoral responses in diabetes mellitus have described essentially no significant abnormalities (8). Cellular immunologic responses have been virtually neglected, but depressed reactivity of diabetic human lymphocytes to phytohemagglutinin stimulation has been reported (9). In experimental animals we have recently observed marked suppression of the cell-mediated granulomatous response to parasite eggs trapped in the livers of mice with schistosomiasis mansoni, associated with striking amelioration of hepatosplenic disease (10). The

present report is concerned with a more precise delineation of suppression of several cell-mediated immunologic responses in chemically induced (streptozotocin and alloxan) and genetically diabetic mice (db/db).

METHODS

Animals. Female Swiss albino mice weighing 20–22 g obtained from Carworth Div., Becton, Dickinson & Co., New City, N. Y., were used for induction of experimental diabetes by streptozotocin and alloxan. Genetically diabetic mice C57BL/KsJ-db (genotype, db/db) and their control littermates (db/+) were obtained from Jackson Memorial Laboratories, Bar Harbor, Maine. Inbred mice (C57BL/6J and A/J) for allograft studies were also obtained from the latter source. All mice were maintained on a standard laboratory diet (Ralston Purina Co., St. Louis, Mo.) and water ad libitum.

Drugs for induction of diabetes. Streptozotocin (lot no. 10518-GGS-37U-9889) was provided by Dr. William Dulin of the Upjohn Company, Kalamazoo, Mich.; it was freshly dissolved before use in citrate buffer, pH 4.5. A group of control mice received nicotinamide (Sigma Chemical Co., St. Louis, Mo.) before the streptozotocin injection. Alloxan (lot no. 691-1) from the Eastman Kodak Co., Rochester, N. Y., was also freshly prepared before use in 0.85% sodium chloride solution.

Chemical procedures. After an overnight fast, blood from the retro-orbital plexus was drawn into heparinized microhematocrit tubes. 25-μl aliquots of blood were assayed for glucose concentration by AutoAnalyzer (11); the remainder of the blood was centrifuged and the plasma was stored at $-20°C$ for subsequent estimations. Diabetic animals which were treated with insulin were fasted for only 4 h before blood samples were taken.

Granuloma formation around Schistosoma mansoni eggs. Groups of five or six mice were used to study the effect of diabetes mellitus of different etiologies on the ability of the host to mount a cell-mediated granulomatous response. 1,500 *S. mansoni* eggs isolated from the livers of mice infected for 8 wk were injected via a tail vein into the pulmonary microvasculature. 8 days later the lungs were removed and preserved in 10% buffered formalin. Sections were cut 5 μm in thickness and stained with hematoxylin and eosin (12). The size of the granulomas around the eggs was determined by measuring their area with a πMC particle measurement computer (Millipore Corp., Bedford, Mass.) as described previously (13). The mean area of approximately 50 lesions from each experimental group was measured and the significance of the differences between groups was assessed by Student's t test. Percent change in the area of inflammation was calculated by subtracting the mean area of the lesions in the experimental animals (area of inflammation minus area of eggs) from that in the control animals, dividing this figure by the mean lesion area in the control mice, and multiplying by 100.

Four groups of streptozotocin-treated mice were studied: (*a*) those injected intravenously with 150 mg/kg body wt, (*b*) those injected intravenously with 175 mg/kg body wt, (*c*) those injected intraperitoneally with 500 mg/kg of nicotinamide before the higher dose of streptozotocin, and (*d*) those injected with 175 mg/kg body wt of streptozotocin and treated with insulin 1 day before egg injection and daily for the subsequent 8 days. Insulin (Isophane Insulin Suspension, U.S.P., M-40, E. R. Squibb & Sons, New York) was administered in a daily subcutaneous dose of 1 U. Controls received intravenous injections of citrate buffer.

Three groups of alloxanized mice were studied: one was injected intraperitoneally with 150 mg/kg body wt, the second with 200 mg/kg body wt, and the third received the higher dose followed by insulin therapy as outlined above. Controls were injected intraperitoneally with 0.5 ml normal saline. Granuloma formation was also studied in six genetically diabetic mice (db/db) and six control littermates (db/+). Blood glucose concentrations and body weights were measured before egg injections, once during the 8-day course of the experiments and when the lungs were removed.

Nonimmunologic granuloma. Divinyl benzene copolymer beads (Bio-Rad Laboratories, Richmond, Calif.) were prepared as described previously (14). 3,000 beads suspended in 0.5 ml of 0.85% sodium chloride solution were injected via a tail vein into six mice rendered diabetic by the intravenous injection of 175 mg/kg body wt streptozotocin and into six untreated control animals. 48 h after the bead injections the lungs were removed and the granulomas measured as described above.

Delayed footpad swelling. This response was studied in a group of mice rendered diabetic by the intravenous injection of 175 mg/kg of streptozotocin and in six untreated controls. Mice were sensitized by the intraperitoneal injection of 1,500 *S. mansoni eggs*. Soluble egg antigens prepared by the method of Boros and Warren (15), equal to 10 μg protein in 0.03 ml phosphate-buffered saline, were injected into the right hind footpad and an equal amount of the buffer into the left hind footpad. 24 h later the lesion was measured by a micrometer (Lufkin Rule Co., Saginaw, Mich.) to 1/100th of a millimeter. The mean difference in thickness between the right and left footpads was taken as the net swelling.

Skin allografts. Grafting of C57BL/6J skin from normal mice onto A/J mice rendered diabetic by the intravenous injection of 100 mg/kg of streptozotocin (the low dose was due to the sensitivity of these mice to the drug) and onto nondiabetic A/J mice was done according to the method described by Billingham (16). Casts were removed on the 8th day and survival of grafts was followed daily by using the criteria of Monaco et al. (17).

RESULTS

Granuloma formation in streptozotocin-treated mice. The mean fasting blood glucose in control animals was 75 mg/100 ml, and that in the animals treated with 150 and 175 mg/kg body wt streptozotocin was, respectively, 171 and 260 mg/100 ml (Table I). Prior injection of nicotinamide protected the animals from the diabetogenic effect of streptozotocin (Table I). Blood glucose determinations 4 and 8 days after the intravenous injection of *S. mansoni* eggs showed no significant differences from preinjection values (Table I). The severely diabetic group treated with insulin had blood glucose concentrations of 248 mg/100 ml before treatment, but during insulin therapy the levels (after only 4 h of fasting) were 146 and 134 mg/100 ml at 4 and 8 days, respectively (Table I).

The control mice increased their body weight 7.3% during the 8-day experimental period. This contrasted with a loss of 7.9% in the group injected with 175 mg/kg of streptozotocin. Treatment of these diabetic

TABLE I

Fasting Blood Glucose Concentrations and Granuloma Formation around S. mansoni Eggs Injected into the Pulmonary Microvasculature of Mice with Streptozotocin-Induced Diabetes Mellitus Including the Effect of Prior Treatment with Nicotinamide and Subsequent Treatment with Insulin

Experimental group	Mean±SE fasting blood glucose			Mean±SE granuloma area (Day 8)	Change in % area of inflammation (Day 8)
	Before egg injection	After egg injection			
		Day 4	Day 8		
	mg/100 ml			*μm² × 10³*	
Control, untreated (5)*	72±4	85±4	79±7	14.3±0.9	0
Streptozotocin, 150 mg/kg (6)	171±17	162±21	159±19	12.1±1.0	−20
Streptozotocin, 175 mg/kg (9)	260±18	281±31	266±22	6.9±0.6	−68
Nicotinamide + streptozotocin, 175 mg/kg (6)	90±3	78±5	85±4	14.9±0.9	+6
Streptozotocin, 175 mg/kg +insulin (6)	248±31	146±20	134±11	16.1±3.2	+17

* The number of animals in each experimental group is shown in parentheses.

mice with insulin, however, resulted in a weight gain of 8.1% during the period of the experiment.

The mean granuloma area around the schistosome eggs 8 days after their injection into the lung arterioles is shown in Table I. The area of inflammation around the eggs was 20% less in the moderately hyperglycemic mice and 68% less in the severely hyperglycemic animals, the latter value being significantly smaller than the mean granuloma size in the control animals ($P < 0.01$). Prior injection of nicotinamide not only protected the animals against the diabetogenic effect of streptozotocin, but it completely prevented the suppression of granuloma formation (Table I). Insulin therapy of the severely diabetic mice reversed the inhibition of granulomatous inflammation (Table I).

Granuloma formation in alloxan-treated mice. Doses of alloxan of 150 and 200 mg/kg body wt induced marked rises in mean fasting blood glucose concentrations to 252 and 400 mg/100 ml, respectively, which were maintained throughout the duration of the experiment (Table II). In the mice injected with the higher dose of alloxan, insulin treatment lowered the blood glucose values from 425 to 171 mg/100 ml at day 4 and 162 mg/100 ml at day 8 after intravenous injection of the eggs (Table II).

Control mice gained 7.3% of their body weight in the 8 days following egg injection while diabetics lost 5.3%, but diabetics treated with insulin gained 17.3%.

Suppression of the granulomatous response to schistosome eggs in the mice treated with both doses of alloxan was highly significant ($P < 0.001$). Again, the insulin-treated diabetic animals recovered their ability to respond to *S. mansoni* eggs with a granulomatous response not significantly different from that of control mice (Table II).

Granuloma formation in spontaneously diabetic mice. Genetically diabetic animals (db/db) and their normal littermates (db/+) had mean blood glucose concentrations before egg injection of 481 and 97 mg/100 ml, respectively (Table III). No significant changes in the

TABLE II

Fasting Blood Glucose Concentrations and Granuloma Formation around S. mansoni Eggs Injected into the Pulmonary Microvasculature of Mice with Alloxan-Induced Diabetes Mellitus Including Subsequent Treatment with Insulin

Experimental group	Mean±SE fasting blood glucose			Mean granuloma area (Day 8)	Change in % area of inflammation (Day 8)
	Before egg injection	After egg injection			
		Day 4	Day 8		
	mg/100 ml			*μm² × 10³*	
Control, untreated (6)*	75±4	69±5	82±3	13.1±1.8	0
Alloxan, 150 mg/kg (6)	252±40	224±30	231±38	8.3±1.0	−65
Alloxan, 200 mg/kg (9)	401±35	407±27	449±39	6.3±0.5	−70
Alloxan, 200 mg/kg +insulin (5)	425±32	171±26	162±24	12.1±0.8	−10

* The number of animals in each experimental group is shown in parentheses.

TABLE III

Fasting Blood Glucose Concentrations and Granuloma Formation around S. mansoni Eggs in Genetically Diabetic Mice (db/db) and Their Nondiabetic Littermates (db/+)

Experimental group	Mean±SE fasting blood glucose			Mean±SE granuloma area (Day 8)	Change in % area of inflammation (Day 8)
	Before egg injection	After egg injection			
		Day 4	Day 8		
	mg/100 ml			*$\mu m^2 \times 10^3$*	
db/+ (6)*	97±5	101±6	110±5	35.4±3.4	0
db/db (6)	481±27	549±38	454±28	10.8±1.0	−77

* The number of animals in each experimental group is shown in parentheses.

blood glucose values were seen at 4 and 8 days after egg injection (Table III). In the genetically diabetic mice the mean granuloma area of approximately 11 $\mu m^2 \times 10^3$ was 77% smaller than that in the control db/+ littermates of 35 $\mu m^2 \times 10^3$ ($P < 0.001$) (Table III, Fig. 1).

Foreign body granuloma. The peak inflammatory response to divinyl benzene copolymer beads, which occurs at 48 h, reached 4.8±0.4 $\mu m^2 \times 10^3$ in the untreated control animals. In a group of streptozotocin-diabetic mice (fasting blood sugar, 266±17 mg/100 ml) the mean granuloma area was 5.2±0.5 $\mu m^2 \times 10^3$. The difference between these groups was not statistically significant.

Delayed footpad swelling. The mean net increase in footpad swelling to soluble *S. mansoni* egg antigens in egg-sensitized control animals (fasting blood glucose,

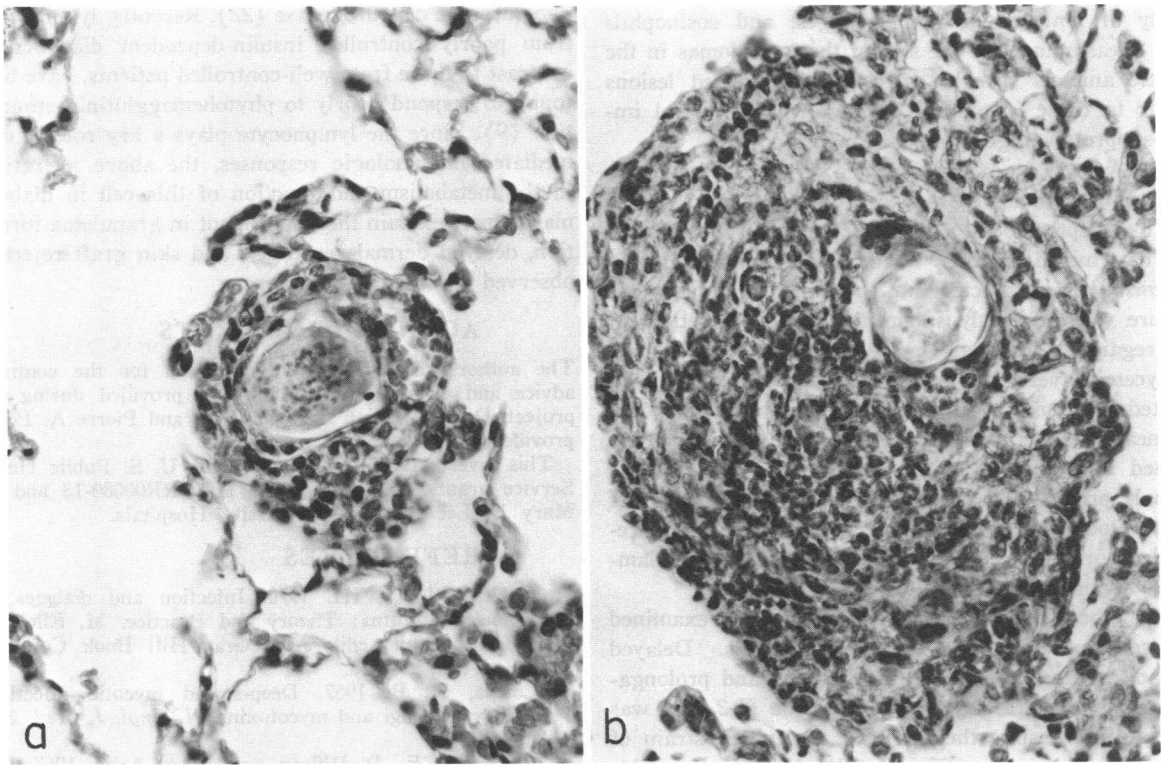

FIGURE 1 Representative granulomas* around *S. mansoni* eggs 8 days after their injection into the pulmonary microvasculature of (*a*) genetically diabetic mice (db/db) and (*b*) their nondiabetic littermates (db/+). * Mean granuloma area determined, sections searched for granuloma representative of the mean, and granuloma marked and photographed.

81±4 mg/100 ml) was 0.42±0.03 mm, but in a group of egg-sensitized, streptozotocin-induced diabetics (fasting blood sugar, 288±18 mg/100 ml) it was 0.05±0.03 mm. The difference between the groups was highly significant ($P < 0.001$).

Allograft survival. The mean allograft survival time in control A/J animals with fasting blood glucose concentrations of 67±5 mg/100 ml was 10.2±0.5 days. In the group of mice with moderate, streptozotocin-induced diabetes (fasting blood glucose 182±19 mg/100 ml) mean allograft survival was prolonged to 14.4±0.4 days ($P < 0.01$).

DISCUSSION

In the course of investigations of the effect of diabetes mellitus on the host-parasite relationship in murine schistosomiasis mansoni, we observed marked amelioration of hepatosplenic disease (10). The principal pathological lesion in schistosomiasis is the host granulomatous response to the parasite eggs trapped in the liver which has been shown to be an immunologic reaction of the cell-mediated type (12, 18). The *S. mansoni* egg granulomas which form slowly on primary exposure (peaking at 16 days) and show anamnestic reactivity on secondary and subsequent exposures are composed largely of lymphocytes, macrophages, and eosinophils (12). Measurement of the size of the granulomas in the diabetic animals revealed markedly suppressed lesions similar to those in animals treated with powerful immunosuppressive agents (19, 20).

In the present study we examined several different cell-mediated immunologic responses in mice rendered diabetic by streptozotocin or alloxan and those with genetically determined diabetes. Granuloma formation around *S. mansoni* eggs injected into the pulmonary microvasculature was uniformly suppressed in the diabetic animals regardless of its etiology. A moderate degree of hyperglycemia averaging around 160 mg/100 ml was associated with only a 20% reduction in granuloma size, but mean glucose values of 225 mg/100 ml or greater resulted in a diminution of inflammation of 65–77%. Animals protected against the hyperglycemic effect of streptozotocin by a prior injection of nicotinamide responded normally to the egg injection with large inflammatory lesions.

Two other cell-mediated responses were examined in streptozotocin-induced diabetic animals. Delayed footpad swelling was markedly inhibited, and prolongation of skin allograft survival across the *H-2* area was demonstrated despite the fact that the inbred strain of mice used in these experiments was highly susceptible to streptozotocin-induced diabetes and had relatively modest hyperglycemia.

While all of the above responses are immunologic in nature and are dependent on intact cell-mediated mechanisms, the foreign-body granuloma is nonimmunologic, being related to activation of chemical mediators of inflammation (14). The reactions around the beads peak in 48 h, show no anamnestic reactivity, and are composed initially largely of neutrophils which are later succeeded by macrophages (14). Streptozotocin-induced diabetes had no effect on the host reaction to plastic beads injected into the pulmonary microvasculature. This dissociation in the effect of immunosuppressive agents on the schistosome egg and plastic bead granulomas has been reported previously with neonatal thymectomy (19), antilymphocyte serum (20), and a new immunosuppressive drug which is specific for cell-mediated reactions, niridazole (13).

The return to normal of the host granulomatous response to *S. mansoni* eggs in diabetic animals treated with insulin suggests that the deficiency in cell-mediated responses may have a reversible metabolic basis. Brody and Merlie (21) examined the metabolism of lymphocytes from diabetic patients in vitro and demonstrated total and proportional decreases in the amounts of glucose passing through the hexose-monophosphate shunt. Evidence from alloxan-diabetic rats suggests that such changes may be due to a deficiency in the enzyme 6-phosphogluconate dehydrogenase (22). Recently, lymphocytes from poorly controlled insulin-dependent diabetics, in contrast to those from well-controlled patients, have been found to respond poorly to phytohemagglutinin stimulation (9). Since the lymphocyte plays a key role in cell-mediated immunologic responses, the above alterations in the metabolism and function of this cell in diabetes may in part explain the impairment in granuloma formation, delayed dermal reactivity, and skin graft rejection observed in these investigations.

ACKNOWLEDGMENTS

The authors thank Dr. B. R. Landau for the continual advice and encouragement which he provided during this project. Douglas Elliot, Earlene Moss, and Pierre A. Peters provided expert technical assistance.

This investigation was supported by U. S. Public Health Service grants AI 08163 and 2 M01 RR00080-13 and the Mary B. Lee Fund of University Hospitals.

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