Immune Responsiveness and Phagocytic Activity of Macrophages in Streptozotocin (SZ)-induced Diabetic Mice

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Abstract

We succeeded in inducing different severities of diabetic state in C3H male mice by repeated intraperitoneal injections of various doses of SZ. SZ-induced diabetic mice were devided into four groups as follows: Group A, B, C and D. SZ, respectively, 3, 5 doses of 45 mg/kg, 5 doses of 60 mg/kg on consecutive days and one of a dose of 200 mg/kg BW. The degree of hyperglycemia and glycosuria were mild in group A and D. Group B was moderate and group C severe with ketonuria and loss of body weight. We investigated the immune response to anti-sheep red blood cells (SRBC) and the phagocytic activity of macrophages in the above mentioned various SZ-induced mice. Antibody forming activities (values of anti-SRBC plaque-forming cells (PFC) and serum agglutinin) were markedly depressed in all of SZ-diabetic groups. The degree of the suppression of antibody response to SRBC in SZ-diabetic mice corresponded with the severity of the diabetic state (C>B>A=D). However, the phagocytic activity of peritoneal macrophages in SZ-diabetic mice was as high as or higher than that in normal controls, using both latex beads and immune complex as test particles. Moreover, we observed that insulin treatment reversed the defect in the immune response in SZ-diabetic mice. These results indicate that the phagocytic activity of peritoneal macrophages was retained but the antibody response was impaired in the SZ-diabetic mice, and this suggested that the impaired antibody response may be a contributing cause of increased susceptibility to infections in a diabetic state.

It is well known that patients with diabetes mellitus have the increased susceptibility to bacterial and mycotic infections (Ambramson et al., 1967; Louria, 1967). Many investigators have reported that the phagocytic activity of leukocytes in diabetic patients is impaired (Perillie et al., 1962; Bybee and Rogers, 1964; Mowat and Baum, 1971; Bagdade et al., 1978).

With respect to immunologic mechanisms, some investigators suggested suppression of the cell-mediated immune response in diabetic patients, by showing the depression of the lymphocyte response to phytohemagglutinin (Brody and Merlie, 1970; Delespesse et al., 1974; MacCush, 1974). In chemically induced and spontaneously diabetic mice, Mahmoud et al. (1975 and

Received August 23, 1983
1976) showed a definite suppression of cell-mediated immune response to *Schistosoma mansoni* eggs. The same investigators also commented that treatment with insulin reversed such depression in chemically induced diabetic mice (Mahmoud et al., 1976). Bellgrau et al., (1982) observed that spontaneously diabetic BB rats, which had several features in common with type 1 human diabetes, were T lymphocytopenic relative to non-diabetic prone BB rats and their T lymphocytes did not respond on MLR (mixed lymphocyte culture) while producing normal responses to the T cell mitogen concanavalin A. On the other hand, Dolkart et al., (1971) described that alloxan-induced diabetic mice had no significant humoral immune response abnormalities. However, Ishibashi et al., (1980) recently reported that in SZ-diabetic mice the humoral immune response to sheep red blood cells (SRBC) was markedly suppressed while the cell-mediated response was mildly depressed. They also suggested that the insulin-deficient diabetic state caused a depression in the immune responses independently of the direct action of streptozotocin.

However, there has been no reported examining of both the humoral immune response and the phagocytic activity in an experimental diabetic animal. In this study, we performed experiments to investigate the humoral immune response and the phagocytic activity of peritoneal macrophages in SZ-induced diabetic mice.

**Materials and Methods**

**Animals**

Inbred C3H/HeM male mice, aged 8–10 weeks and weighing 25–35 g (supplied from the Institute of Laboratory Animals, Faculty of Medicine, Kyoto University) were used.

**Induction of streptozotocin (SZ) diabetes**

Streptozotocin (Lot. 60273-4) was obtained from Upjohn Company (Kalamazoo, Michigan, USA). In all experiments, we injected SZ in the citrate buffer (pH 4.2) intraperitoneally into mice. SZ-induced diabetic mice were divided into 4 groups as follows: First group (A) of mice was injected daily over 3 days with SZ, 45 mg/kg body weight. Second (B) was injected with SZ, 45 mg/kg over 5 days. Third (C) was injected with SZ, 60 mg/kg over 5 days. Fourth (D) received a single injection of SZ at a dose of 200 mg/kg. Control group (E) was injected with the buffer alone. Bloop samples for glucose determinations were collected at 09.00 to 10.00 h from the paraorbital venous plexus of non-fasting animals, using glass pipettes. Samples were obtained before SZ injection and at frequent intervals afterward until animals were sacrificed for the plaque-forming cells (PFC) assay. Blood glucose levels were determined by the glucose-oxidase method. Test-tapes (Urolabstix-III, Ames Division of Miles-Sankyo Co. Ltd., Tokyo, Japan) were used to test for glycosuria and ketonuria.

**Antibody response to sheep red blood cells (SRBC)**

SRBC were washed three times in saline before immunization. SRBC were injected intraperitoneally at a dose of $5 \times 10^8$ into the SZ-diabetic and the control animals 5 days after the last SZ injection. Anti-SRBC response were determined 5 days after immunization by assaying the number of antibody-forming cells as plaque-forming cells (PFC) in the spleen (Hosono and Muramatsu, 1972) and the titer of anti-SRBC serum agglutination (Muramatsu and Hosono, 1974). Mice receiving saline alone served as controls, but their natural immunity was negligible. Only direct PFC and 2-mercaptoethanol sensitive antibodies (IgM) were detected in these experiments.

**Phagocytic activity**

Since details were described in our previous paper (Nakano et al., 1978a), only brief mention will be made below.

Peritoneal exudate cells (PEC) were obtained from peritoneal cavities of SZ-induced diabetic mice (the 9th day after the last injection) and normal controls using plastic syringes. To adhere macrophages onto glass slips, $1 \times 10^6$ PEC were added to glass petri dishes (60 mm diameter) with rectangular glass cover slips (22 x 10 mm) on the bottoms and were incubated for 24 hours in a CO$_2$ incubater (5 % CO$_2$-95 % air). Phagocytic activities were determined using above described
macrophages. Test particles used were poly-
styrene latex beads (diameter 2.0 μm) and SRBC- anti SRBC rabbit IgG complex (EA). The 1 : 1 mixture of SRBC (E) suspension (10⁷/ml) and anti-E IgG (A), which was obtained one week after intravenous injection of 2×10⁷ E, at a final concentration just below agglutination E was incubated with gentle shaking at 37°C for 15 min. After washing three times with Eagle minimum essential medium (MEM), EA thus prepared was suspended at 10⁸ cells/ml. Ingestion of test particles (at 37°C for 2 hours) into macrophages were evaluated under a negative phase contrast microscope after glutaraldehyde fixation. To examine the ingestion of EA by macrophages, cover slips were dipped into 0.83 % NH₄Cl for 5 minutes to remove any extra-
cellularly attached erythrocytes before fixation. In each test, at least 400 macrophages were ex-
amined. The number of particles ingested per 100 macrophages is expressed as the ingestion index. Percentages of macrophages with particles were also determined.

**Insulin treatment in SZ-diabetic mice**

The following experiments were performed to determine whether insulin treatment could restore the depression of antibody response to SRBC in the diabetic state. Half the diabetic mice (SZ 60 mg/kg BW. daily over five days) were given sub-
cutaneously a single 1 U dose of NPH insulin (Eli Lilly, Indianapolis, IN) daily for 14 days from 11 days after the last SZ injection until the day of detection of hemagglutinin titer. SRBC were injected intraperitoneally at a dose of 5×10⁸ on the 10th day after insulin treat-
ment. Anti-SRBC serum agglutinin was deter-
mined 5 days after immunization.

Statistical analysis of data was performed using Student’s t-test. Mean values were ex-
pressed as mean±SEM.

**Results**

**SZ-induced diabetic mice**

Plasma glucose, urine and body weight were checked daily to assess general animal condition. Plasma glucose and urine in non-
fasting animals were examined and the body weight in those fasting was measured. Fig. 1 illustrates the results of glucose determi-
nations over 15 days. The glucose elevations were observed and continued after SZ ad-
ministration for the following days. The plasma glucose levels in all of SZ treated mice were significantly higher on the 5th day after the last SZ administration than in the controls (p<0.005) (A : 203±3, B : 318±4, C : 320±7, D : 220±10 mg/dl versus Cont. : 135±5 mg/dl. All of the SZ treated mice showed glycosuria 2 days after the last SZ administration. Glycosuria especially in group B and C became markedly positive on the 5th day after the last SZ admini-
stration. But ketonuria showed up only in group C 5 days after the last SZ injection. Also the mean body weight had decreased by 10 % but only in group C and on the 10th day after the last SZ injection. Judging from the above mentioned data, was clas-
sified the severities of the diabetic state in SZ treated mice as follows. Group A and D were mild. Group B was moderate and Group C severe.

**Antibody response to SRBC in SZ-induced diabetic mice**
Table 1. Antibody response to SRBC in SZ-diabetic mice

<table>
<thead>
<tr>
<th>Group</th>
<th>SZ-injection (mg/kg, IP.)</th>
<th>No. of spleen cells ($\times 10^6$)</th>
<th>PFC/spleen</th>
<th>PFC/10^6 spleen cells</th>
<th>HA titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>45 $\times$ 3</td>
<td>5.7 $\pm$ 0.2</td>
<td>10,755 $\pm$ 74.4</td>
<td>717 $\pm$ 86*</td>
<td>$8.8 \pm 0.3$</td>
</tr>
<tr>
<td>B</td>
<td>45 $\times$ 5</td>
<td>5.2 $\pm$ 0.2**</td>
<td>4,000 $\pm$ 318</td>
<td>267 $\pm$ 37$\dagger$</td>
<td>$6.5 \pm 0.3$</td>
</tr>
<tr>
<td>C</td>
<td>60 $\times$ 5</td>
<td>4.7 $\pm$ 0.1$\dagger$</td>
<td>1,778 $\pm$ 206</td>
<td>119 $\pm$ 24$\dagger$</td>
<td>$4.0 \pm 0\dagger$</td>
</tr>
<tr>
<td>D</td>
<td>200 $\times$ 1</td>
<td>3.5 $\pm$ 0.7$\dagger$</td>
<td>10,721 $\pm$ 970</td>
<td>715 $\pm$ 112*</td>
<td>$8.5 \pm 0.3$</td>
</tr>
<tr>
<td>E</td>
<td>buffer (control)</td>
<td>6.6 $\pm$ 0.4</td>
<td>18,417 $\pm$ 458</td>
<td>1,228 $\pm$ 168</td>
<td>$11.0 \pm 0$</td>
</tr>
</tbody>
</table>

The number of spleen cells was significantly lower in diabetes except group A than it was in the normal controls. The number of spleen cells in group D (a single injection of SZ, 200 mg/kg) was especially the lowest among all of the diabetic groups. Both the number of PFC per spleen and 10^6 spleen cells and hemagglutinin titer in diabetes were extremely reduced as compared with that in normal controls. Antibody production in group C was most remarkably depressed in all of the SZ-diabetic groups. However in group D, whose number of spleen cells was the lowest among all of the diabetic groups, the degree of the suppression of antibody response was as mild as in group A. These data indicate that the degree of the suppression of antibody response to SRBC in the SZ-diabetes corresponded with the severities of the diabetic state previously described.

Phagocytic activity

The number of peritoneal macrophages in each group was 1.8–2.0 $\times$ 10^6/mice and the difference between diabetic and normal mice was not so conspicuous (data not shown). Fig. 2 shows that the ingestion of EA and latex beads by diabetic and normal peritoneal macrophages. Ingestion indices for EA and latex were not lower in the diabetic groups than those in the controls. Moreover, the phagocytic activity in group C rather increased when compared with that in the normal controls. These results indicate that the phagocytic activity of peritoneal macrophages in a diabetic state was not reduced but as great as or greater than

Fig. 2. Phagocytic activity of resident peritoneal macrophages from SZ-diabetic and normal mice. A, B, C and D are diabetic groups (detail described Materials and Methods). E is normal controls. Each group is composed of ten animals. Height of each column represents ingestion index of macrophages in each group. Figures on columns are percentages of macrophages carrying EA and latex.
Influence of insulin treatment on the antibody response in the diabetic mice

The mean blood glucose levels observed in the insulin-treated diabetic mice are shown in Fig. 3. After insulin treatment, the blood glucose levels of treated diabetic mice had decreased significantly (\(p<0.005\)) compared with the values of untreated diabetic mice. But complete recovery of the hyperglycemia state of diabetic mice by insulin treatment (a single 1-U dose of NPH insulin/mouse) was not possible. However, ketonuria could be improved by insulin treatment. Moreover, the weight loss was partially ameliorated on the 12th day after insulin treatment (data not shown). Then, the results in Table 2 clearly show that insulin treatment restored the antibody response to a nearly normal level. These results indicate that insulin treatment reversed the depressed immune response in the SZ induced diabetic mice by improving the metabolic state in diabetic mice.

Discussion

Like and Rossini (1976) reported that multiple injections with low doses of SZ in mice produced diabetes mellitus. In this our study, we investigated antibody response to SRBC and phagocytic activity of peritoneal macrophages in the SZ diabetic mice inducing it according to the method of Like & Rossini.

In this study, we showed that hyperglycemia and glycosuria were observed in all of the SZ treated groups two days after the last injection. The degree of hyperglycemia and glycosuria was mild in group A (injected on consecutive days with SZ at 3 doses of 45 mg/kg) and D (at a dose of 200 mg/kg once). On the other hand, ketonuria and weight loss were observed only in group C (injected with SZ at 5 doses of 60 mg/kg on consecutive days) 5 days after the last injection.

We observed that antibody forming activities in experimental mice were depressed according to the severities of the diabetic state. In group C, the number of PFC and the titer of HA were one tenth and one hundred twenty-eighth, respectively, as compared with the normal controls. On the other hand, in group A and D with a mild diabetic state, the number of PFC was half and the titer of HA was one quarter as compared to the controls. Recently, Nichols et al. (1978 and 1979) reported that SZ-induced diabetes in mice was associated with a large
decrease in the size and the nucleated cell number of lymphoid organs by the direct immunsuppressive effects of SZ rather than the SZ-induced diabetic state. It is doubtful whether the depressed immune response in SZ diabetic mice depends on the direct immunosuppressive effects of SZ or a deficiency of insulin in our system. We observed a marked decrease in the number of spleen cells in SZ-treated mice immunized with SRBC. However in group D, whose number of spleen cells was the lowest among all of the diabetic groups, the degree of suppression of antibody response to SRBC was as mild as that seen in group A whose number of spleen cells was not low compared with the values of normal controls. Moreover, we observed that treatment with insulin reversed the defects in the immune response in the SZ-diabetic mice. Pavelic et al. (1978) reported that in alloxan-induced diabetic mice, insulin treatment reversed defects in lymphoid organs. Recently, Ishibashi et al. (1980) also reported that treatment with insulin reversed the depression of antibody production and proliferation, using SZ-diabetic mice. Thus, it seemed logical to suppose that the SZ-induced chronic diabetic state, rather than the direct effect of SZ, caused such a defect in the immune response.

In this study, we also investigated the phagocytic activity of peritoneal macrophages in SZ diabetic mice under in vitro condition. The test particles used were polystyrene latex beads and immune complex (EA). EA was phagocytosed via Fc receptors on macrophages but latex beads were not ingested via specific receptors (Nakano et al., 1978a). We observed that the phagocytic activity of macrophages for both particles in diabetic mice was as high as or higher than that in normal mice. These results were interesting when referring to our previous reports (Nakano et al., 1978b). We have shown that newborn mice under immunological underdevelopment had a higher phagocytic activity than that of adult mice be examining ontogeny of murine macrophage function. Namely, under immunological immaturity macrophages play a chief role against inflammation and hold a strong phagocytic activity (Nakano et al., 1978b). In general, impaired phagocytosis by polymorphonuclear leukocytes was emphasized in laboratory animals (Wertmann and Henney, 1962; Drachman et al., 1966) and humans (Perillie et al., 1962; Bybee and Rogers, 1964; Mowat and Boum, 1971; Bagdade et al., 1978) with diabetes mellitus. However, phagocytosis by macrophages in both experimental animals and humans with diabetes had not been extensively investigated. There have been some reports about phagocytosis by stationary macrophages of the reticuloendothelial system (RES) in both diabetic subjects. Cruikshank (1954) reported that there were no changes in the phagocytic clearance of pneumococcal bacteria from the blood following intravenous administration in alloxan-induced diabetic rabbits compared with that in normal controls. Berker and Sherman (1974) was unable to show any changes in phagocytosis of RES among diabetes, with or without retinopathy and non-diabetics, by examining intravenous doses of radioiodinated microaggregated albumin that approximated those used for the measurement of liver blood flow. Schneider et al. (1980) showed that RES phagocytosis by liver and spleen macrophages was significantly reduced in alloxan-induced diabetic rats. Drivas and Wardle (1978) pointed out that the diabetic patients with nephropathy or retinopathy had impaired RES function. They suggested that a dysfunction of the phagocytic cells had implications for the cellular pathology of microangiopathy. On the contrary, Cornell (1981 and 1982) recently reported that RES hyperphagocytosis was observed in SZ-induced diabetic rats, using both non-physiologic and physiologic test particles. He also showed (1981) that chronic regulation of plasma glucose levels
by insulin treatment, but not antibiotic treatment, of diabetic rats reversed the enhanced phagocytosis of colloidal carbon. Thus, the reports about the phagocytic activity of macrophages in both diabetic subjects were controversial. We suppose that the differences of species of animals, experimental procedures and severities of the diabetic state might be some reasons for these discrepancies.

In conclusion, we found that the phagocytic activity of peritoneal macrophages was retained but the antibody response was impaired in the SZ-diabetic mice. We think that the antibody response, which has been impaired, may contribute to one of the causes of increased susceptibility to infections in a diabetic state. Further experiments must be conducted to identify the cell type or types responsible for the impaired immune response in the SZ-diabetic mice.

References


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