Impaired Response of Lymphocytes from Non-Insulin-Dependent Diabetics to Staphage Lysate and Tetanus Antigen

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We examined the blastogenic responses of lymphocytes to *Staphylococcus aureus* antigen in 49 uninfected non-insulin-dependent diabetic subjects and in 56 uninfected nondiabetic subjects. We found a significantly decreased response to this antigen in the diabetic patients ($P < 0.05$). Diabetics whose blood sugar levels were <150 mg/dl showed significantly decreased responses, as did diabetics whose blood sugar levels were >200 mg/dl. The use of fetal calf serum in place of autologous plasma did not correct the defect. In a smaller number of patients, we observed a decreased response to tetanus toxoid, which was significant ($P < 0.05$). The response to phytohemagglutinin was not significantly different from normal controls ($P > 0.4$). These results indicated that non-insulin-dependent diabetics, as well as insulin-dependent diabetics (previously reported), show decreased responses to *S. aureus* antigen and to tetanus toxoid. This defect apparently is not related to blood glucose levels or other serum factors.

Impaired cell-mediated immunity (CMI) in diabetics is being reported with increasing frequency. Ragab et al. found no differences between normal individuals and diabetic individuals in good metabolic control when they compared the responses of peripheral blood lymphocytes (PBL) to either phytohemagglutinin (PHA) or *Candida* antigen (18). Other studies, performed when diabetic subjects were in poor metabolic control, showed a depressed lymphocyte response to PHA (4). Decreased responses to staphylococcal antigen (SPL) and streptokinase-streptodornase have been demonstrated in the PBL of insulin-dependent diabetic subjects and in a small number of non-insulin-dependent diabetic subjects (5, 17).

The immune responses of hosts to staphylococcal infections are cell mediated at least in part (11, 12). Mudd et al. used SPL to demonstrate that 94% of normal individuals had a delayed-type hypersensitivity reaction at 48 h (15), and Dean et al. reported that the PBL from 95% of the normal individuals whom they studied reacted to SPL in the lymphocyte transformation assay (7).

The purpose of this study was to extend our previous investigations of the PBL response to SPL to non-insulin-dependent diabetic subjects. At the same time, we investigated the response of PBL from diabetic subjects to tetanus toxoid (TT), a bacterial antigen which has been used in many studies of T-cell function, and to the mitogen PHA, to which we had observed normal responses in insulin-dependent diabetics.

MATERIALS AND METHODS

Subjects. Diabetics were selected by previously described criteria (5). Briefly, these individuals were patients who came to the ambulatory care clinic for routine visits. The age range was 37 to 82 years. Normal subjects were chosen from among our hospital personnel and patients from the general medical clinic who did not have any illnesses associated with depressed CMI; the age range of these subjects was 21 to 68 years. Neither patients nor normal subjects were on medications known to suppress lymphocyte function. All of the diabetics were non-insulin dependent and in good metabolic control (blood sugar levels, less than 275 mg/dl; urine sugar levels, 2+ or less; no acetone). Usually, blood sugar levels were determined 2 or 3 days before the clinic appointment. Hemoglobin A1-C assays were done on the same blood specimens that were used for the lymphocyte transformation test.

Cell culture. Lymphocytes were separated from defibrinated venous blood as described previously (13). Briefly, about 30 ml of blood was defibrinated with glass beads in an Erlenmeyer flask. After the clot was removed, 5 ml of 5% dextran was added, and the blood was transferred to a 50-ml centrifuge tube and allowed to settle for 1 to 1.5 h at 37°C. The supernatant plasma containing the leukocyte suspension was removed and centrifuged, and the cells in the pellet were washed twice and then suspended in Dulbecco modified Eagle medium (GIBCO Laboratories, Grand Island, N.Y.). The cells were cultured in flat-bottomed microtiter plates (M. A. Bioproducts, Walkersville, Md.) to a concentration of 2×10^5 cells per culture in Dulbecco modified Eagle medium supplemented with either 10% fetal calf serum (FCS) or 10% autologous plasma (AP). Then we added SPL (Staphage Lysate; Delmont Laboratories, Swarthmore, Pa.) to a final concentration of 10% (140 ng of protein per well) or

solubilized TT (Wyeth Laboratories, Marietta, Pa.) to a final concentration of 15% (2 μ g of protein N per well). All cultures were done in triplicate. Microtiter plates were covered and incubated in a humidified 5% CO₂ incubator at 37°C; 1.0 μ Ci of [³H]thymidine (Schwarz/Mann, Orangeburg, N.Y.) per 0.2 ml of culture was added after 5 or 6 days (for SPL) or after 6 days (for TT). The cultures were returned to the incubator for 3 h, and then the cells were harvested by successive washing with a Mash II harvester (M. A. Bioproducts), using Reeve-Angel type 934 AH glass fiber filters. Then filters containing precipitates were placed into scintillation vials and allowed to dry, and 0.5 ml of hydroxide of hyamine (Packard Instrument Co., Inc., Rockville, Md.) was added. The vials were incubated at 37°C overnight, and then 15 ml of scintillation fluid was added. Radioactivity was measured with a Tri-Carb scintillation spectrometer (Packard Instrument Co.). The arithmetic means of the triplicate samples were determined, and the results were expressed as counts per minute of stimulated cultures $\pm$ the standard error. Repeated tests of the same three normal subjects were performed to assure consistent results from different lots of reagents and other aspects of the culture technique.

Time and dose-response curves. Time and dose-response curves for SPL were determined by incubating lymphocytes from several normal and diabetic subjects with different dilutions of the stock SPL preparation and harvesting the cells on different days; 5 and 6 days of incubation were optimal for the largest number of subjects in both groups. Figure 1 shows a representative time curve from several experiments, and Fig. 2 shows typical dose-response curves for diabetics and normal individuals. Optimal responses for both groups occurred at doses of SPL ranging from 3 to 10% (vol/vol) SPL stock solution. A sample containing 10% SPL stock solution was used in the experiments described below. Dose-response and time curves were determined for PHA and TT. The optimal

amount of PHA was 0.1 ml of a 1:100 dilution of the PHA stock solution, and the optimal amount of TT ranged from 10 to 20% (vol/vol). In the experiments with TT, most subjects showed good proliferative responses on days 6 through 8. We chose day 6 so that the cells for these experiments could be harvested with the cells for the SPL experiments. Thus, one set of unstimulated controls was used for both antigens.

Statistical analyses. Statistical analyses were performed by the method of Oppenheim et al. (16). The data were normalized by logarithmic conversion of the arithmetic means of the total counts per minute of the triplicate samples and then analyzed by the Student *t* test (1).

RESULTS

Blastogenic responses of normal and non-insulin-dependent diabetic subjects to SPL and FCS and to SPL and AP. Initially, PBL from 22 normal subjects and 20 diabetic subjects were used. Cultures containing SPL as the stimulant and 10% FCS in the nutrient medium were pulsed and harvested on day 5. The level of [³H]thymidine incorporation into the DNA of the PBL from the diabetic subjects was $3,090 \pm 577$ cpm (mean $\pm$ standard error), compared with $10,544 \pm 1,494$ cpm for the nondiabetic subjects ($P < 0.02$).

Since the optimal proliferative responses of

FIG. 1. Incorporation of [³H]thymidine into lymphocytes incubated with SPL for different time periods.

FIG. 2. Incorporation of [³H]thymidine into lymphocytes from normal and diabetic subjects exposed to different concentrations of SPL. Symbols: $\circ$, non-insulin-dependent diabetic subjects; $\bullet$, normal subjects.

stimulated PBL in some subjects were observed on day 6, we compared additional groups of subjects whose lymphocytes were harvested on that day. PBL from 34 normal and 29 diabetic subjects were cultured with SPL for 6 days. The level of [^3H]thymidine incorporation into the DNA of the PBL from the diabetic subjects was $13,893 \pm 1,753$ cpm, compared with $26,230 \pm 3,025$ cpm for the normal subjects ($P < 0.05$).

When AP was used in the nutrient medium, the results were similar. We compared the responses of PBL from 15 diabetic subjects and 18 normal subjects to SPL on day 5; with the PBL from the diabetic subjects $14,090 \pm 3,120$ cpm were incorporated, and with the PBL from the nondiabetic subjects $51,592 \pm 7,590$ cpm were incorporated ($P < 0.025$). On day 6, the value for the PBL from 16 diabetic subjects was $32,794 \pm 6,273$ cpm, and we found a value of $89,480 \pm 8,416$ cpm for 25 normal subjects ($P < 0.005$) (Table 1).

Blastogenic responses of normal and non-insulin-dependent diabetic subjects to TT. Lymphocytes from normal and non-insulin-dependent diabetic subjects were cultured with TT and either FCS or AP for 6 days. Gottlieb et al. considered $<10,000$ cpm as a low-responder state to TT when human serum was used in the nutrient medium (10). When we used AP, 20 of 22 (90%) normal subjects showed responses of $>10,000$ cpm to TT, which was similar to the results of Gottlieb et al. with normal subjects. Only 11 of 29 (38%) diabetic subjects showed responses of $>10,000$ cpm. The blastogenic re-

sponses of the normal subjects ranged from 2,948 to 126,351 cpm. The range for the diabetics was 124 to 113,566 cpm. The mean blastogenic response of 29 diabetics was $5,195 \pm 751$ cpm, whereas that of 22 normal subjects was $30,076 \pm 3,922$ cpm ($P < 0.001$). When we used FCS, the mean value for the 29 diabetics was $3,388 \pm 444$ cpm, and the mean value for the 22 normal subjects was $7,081 \pm 976$ cpm ($P < 0.05$) (Table 2). The relationship between blastogenic response to TT and prior exposure of the lymphocytes to the antigen is not clear (10). Therefore, we eliminated those subjects who could not recall receiving TT in the past and compared only those who remembered that they had received TT. When this was done, the results in AP were significantly different ($36,459 \pm 3,552$ cpm for the 20 normal subjects and $9,389 \pm 2,190$ cpm for the 9 diabetics; $P < 0.005$). In FCS the value for 20 normal subjects was $8,177 \pm 1,149$ cpm, and the value for 10 diabetics was $9,154 \pm 1,936$ cpm. Other workers have suggested that AP is more useful than FCS in showing differences in responses to TT in different groups (10), and our data are consistent with this suggestion.

Blastogenic responses of diabetic patients with blood glucose levels of <150 and >200 mg/dl to SPL. We found no difference between the responses of non-insulin-dependent diabetic subjects who had blood glucose levels of <150 mg/dl and the responses of diabetics whose blood glucose levels were >200 mg/dl. When SPL and FCS were used, the incorporation into the PBL

TABLE 1. Blastogenic responses of lymphocytes from normal and non-insulin-dependent diabetic subjects to SPL in media containing FCS and AP

Group ^a	No.	Median age (years)	Type of serum or plasma	Day of harvest	Radioactivity (cpm) in:		Significance (P)	
					Control PBL	SPL-treated PBL	Control PBL	SPL-treated PBL
Normal	22	37 (22-65) ^b	FCS	5	$1,170 \pm 160^c$	$10,544 \pm 1,494^c$	0.20	0.02
Non-insulin-dependent diabetic	20	62 (49-72)	FCS	5	734 ± 121	$3,090 \pm 577$		
Normal	34	38 (21-68)	FCS	6	$3,632 \pm 320$	$26,230 \pm 3,025$	0.10	0.05
Non-insulin-dependent diabetic	29	57 (37-82)	FCS	6	$2,750 \pm 234$	$13,893 \pm 1,753$		
Normal	18	37 (22-65)	AP	5	903 ± 136	$51,592 \pm 7,590$	0.10	0.025
Non-insulin-dependent diabetic	15	60 (49-60)	AP	5	559 ± 86	$14,090 \pm 3,120$		
Normal	25	38 (21-66)	AP	6	$1,623 \pm 160$	$89,480 \pm 8,416$	0.30	0.005
Non-insulin-dependent diabetic	16	55 (38-82)	AP	6	$1,293 \pm 201$	$32,794 \pm 6,273$		

^a The groups of normal and diabetic subjects harvested on day 5 were made up of different individuals than the groups harvested on day 6.

^b The numbers in parentheses are ranges.

^c Mean $\pm$ standard error.

TABLE 2. Blastogenic responses of lymphocytes from normal and non-insulin-dependent diabetic subjects to TT in media containing FCS and AP^a

Group	No.	Median age (years)	Type of serum or plasma	Radioactivity (cpm) in:		Significance (<i>P</i>)	
				Control PBL	TT-treated PBL	Control PBL	TT-treated PBL
Normal	22	37 (21–66) ^b	FCS	3,445 ± 425 ^c	7,081 ± 976 ^c		
Non-insulin-dependent diabetic	29	57 (26–82)	FCS	1,972 ± 245	3,388 ± 444	0.05	0.05
Normal	22	37 (21–66)	AP	1,462 ± 173	30,076 ± 3,922		
Non-insulin-dependent diabetic	29	57 (26–82)	AP	1,248 ± 137	5,195 ± 751	0.50	0.001

^a All cultures were harvested on day 6.^b The numbers in parentheses are ranges.^c Mean ± standard error.

from 14 diabetic subjects with blood glucose levels of <150 mg/dl was 4,683 ± 1,038 cpm, compared with 4,267 ± 680 cpm for 20 diabetic subjects whose blood glucose levels were >200 mg/dl (*P* > 0.8). When AP was used, the incorporation into the PBL from eight diabetic subjects with blood glucose levels of <150 mg/dl was 13,643 ± 4,295 cpm, compared with 22,172 ± 3,660 cpm for 22 diabetic subjects whose blood glucose levels were >200 mg/dl (*P* > 0.4) (Table 3). Hemoglobin A1-C levels were determined for 25 patients, and the correlation with the blood sugar levels was very good (*r* = 0.645).

Blastogenic responses of normal and non-insulin-dependent diabetic subjects to PHA and FCS and to PHA and AP. PBL from 18 normal subjects and 17 non-insulin-dependent diabetic subjects were exposed to nutrient medium containing PHA and FCS (Table 4). The level of incorporation of [³H]thymidine into the DNA of the PBL from diabetic subjects was 29,478 ± 5,352 cpm, compared with 26,170 ± 4,038 cpm in the normal subjects (*P* > 0.7). When AP was used, the total counts per minute for the diabetics was 34,578 ± 7,072, compared with 53,199 ± 11,059 cpm for the normal subjects (*P* > 0.4).

Analysis of age factors. The blastogenic responses of lymphocytes to some mitogens de-

crease with age. We compared the responses to SPL in normal subjects who were 20 to 39 years old with the responses of subjects who were 40 to 59 years old. The incorporation value for the 37 subjects who were 20 to 39 years old was 17,689 ± 1,922 cpm, and the value for the 20 subjects who were 40 to 59 years old was 21,286 ± 3,543 cpm (*P* > 0.05). The difference was not significant. Thus, although in general our diabetics were slightly older than our normal subjects, we do not think that this can account for the decreased responses observed in our diabetic population.

Miscellaneous. Since the values for unstimulated cultures from diabetic patients were usually lower than the values for nondiabetic subjects, we compared these data by the Student *t* test. As Tables 1 through 4 show, the differences were not significant. There was no actual relationship between the level of [³H]thymidine incorporation into unstimulated lymphocytes and the level of incorporation into stimulated lymphocytes in either the diabetic group or the nondiabetic group.

DISCUSSION

We observed impaired responses of PBL from non-insulin-dependent diabetic subjects to the

TABLE 3. Blastogenic responses of lymphocytes from non-insulin-dependent subjects with blood glucose levels of <150 and >200 mg/dl to SPL in media containing FCS and AP

Blood glucose level (mg/dl)	No. of subjects	Median age (years)	Type of serum or plasma	Radioactivity (cpm) in:		Significance (<i>P</i>)
				Control PBL	SPL-treated PBL	
<150	14	59 (38–72) ^a	FCS	1,301 ± 228 ^b	4,683 ± 1,038 ^b	
>200	20	61 (42–82)	FCS	551 ± 74	4,267 ± 680	0.8
<150	8	55 (46–69)	AP	492 ± 128	13,643 ± 4,295	
>200	22	59 (42–82)	AP	625 ± 79	22,172 ± 3,660	0.4

^a The numbers in parentheses are ranges.^b Mean ± standard error.

TABLE 4. Blastogenic responses of lymphocytes from normal and non-insulin-dependent diabetic subjects to PHA in media containing FCS and AP^a

Group	No.	Type of serum or plasma	Radioactivity (cpm) in:		Significance (P)	
			Control PBL	PHA-treated PBL	Control PBL	PHA-treated PBL
Normal	18	FCS	925 ± 134 ^b	26,170 ± 4,038 ^b		
Non-insulin-dependent diabetic	17	FCS	1,214 ± 180	29,478 ± 5,352	0.4	0.7
Normal	14	AP	782 ± 122	53,199 ± 11,059		
Non-insulin-dependent diabetic	14	AP	646 ± 110	34,578 ± 7,072	0.4	0.5

^a All cultures were harvested on day 4.^b Mean ± standard error.

bacterial antigens SPL and TT but not to the mitogen PHA.

Patients with either type I (juvenile onset) or type II (adult onset) diabetes have a poor prognosis when they are infected with *Staphylococcus aureus* (6). Because specific hypersensitivity is associated with lowered resistance to *S. aureus* (3, 11, 12), we expected that lymphocytes from diabetics would show greater than normal responses to SPL. We did find one or two patients in both the insulin-dependent diabetic group and the non-insulin-dependent diabetic group who showed responses which were greater than the responses of any of the normal subjects. One such patient whose lymphocytes were retested several weeks later had low responses. However, the majority of our diabetic subjects showed responses which were significantly lower than the responses of our normal controls. More relevant to our data are the findings of Baughn and Bonventre (2), who demonstrated that the type of immunity present after infection with *S. aureus* involves lymphokine production by sensitized lymphocytes and subsequent macrophage activation. These authors suggested that CMI is important in host defense against this organism and that CMI is manifested at the level of phagocytosis. Their data are consistent with those of Rogers and Melly, who suggested much earlier that staphylococci may closely resemble tubercle bacilli in their ability to establish long-term infections in tissues and to survive in host phagocytic cells (21). The defect in lymphocyte reaction to SPL which we observed in the diabetic population could have been associated with decreased clearance of staphylococci from extracellular fluids and might be one explanation for the poor prognosis of diabetic patients who develop staphylococcal infections.

Plouffe et al. (17) reported diminished lymphocyte responses to streptokinase-streptodornase in a small number of non-insulin-dependent dia-

betic subjects, and Fernandes et al. (9) observed diminished CMI in mutant diabetic mice, animals whose disease resembled that of non-insulin-dependent subjects rather than insulin-dependent diabetic humans. These authors suggested that the problem could be related to hyperglycemia, to hyperinsulinemia, to other metabolic factors, or to decreased insulin receptor activity on the lymphocytes. Our data indicate that hyperglycemia is probably not an important factor since diabetic patients whose blood glucose levels were less than 150 mg/dl had no advantage over those whose levels were more than 200 mg/dl. These data are supported by the hemoglobin A1-C levels, which correlated well with blood sugar levels. We have not investigated blood insulin levels, nor have we studied any other metabolic factors. As other workers have suggested, further work should be done to clarify this point.

Since despite fractionation studies it is not clear which cell type is responsible for responses to SPL (7), we tested TT, which has long been used as an indicator of T-cell function. Again, the PBL from the diabetics were less reactive than the PBL from the normal controls. We do not know the reason for the decreased response to SPL and TT. Although it is possible that the problem is intrinsic to diabetic lymphocytes, this is unlikely in view of the normal response to PHA. It is possible that this is a problem of poor recognition of any bacterial antigen, since we now know at least three antigens to which diabetic lymphocytes respond suboptimally. Both SPL and TT are macrophage dependent, so poor antigen processing by macrophages could result in impaired lymphocyte responses. Alternatively, the impaired lymphocyte responses may result in deficient production of macrophage activity factor. In support of the defective macrophage theory, Rodman et al. observed impaired intracellular killing of *Listeria monocytogenes* cells by macrophages from streptozoto-

cin-treated mice (20). Also, footpad swelling after these organisms were injected was much less in the diabetic mice. Mahoud et al. (14) observed no granuloma formation in streptozotocin-treated diabetic mice after injections of *Schistosoma mansoni* antigen into their livers; this was in contrast to non-diabetic mice, in which granuloma formation was rapid and consistent. Both of these studies suggested that there were defects in the diabetic macrophages in the handling of bacterial antigens. To date, because of the difficulty of obtaining sufficient numbers of macrophages from human subjects, these studies have not been performed.

Other possible explanations for the decreased lymphocyte responses to bacterial antigens in diabetics include qualitative or quantitative deficiencies in T-cell subsets. Using monoclonal antibodies, Reinherz et al. (19) defined several subsets of T cells, one of which (OKT₄) proliferated maximally to soluble antigens. This subset was equivalent to the helper cell population. The OKT₈ subset contained the suppressor cells, and as Plouffe et al. (17) suggested, the defect in the diabetic lymphocytes could be related to increased suppressor cell activity. Other observations which may be related to our findings with TT are the recent studies which showed that the blast transformation responses of T lymphocytes could be affected by the existence of B lymphocytes with suppressor activity on T-cell function (8). In this study the authors found no relationship between blast transformation and the degree of the antibody response. Thus, several studies have supported the observation that certain aspects of CMI are diminished in individuals with diabetes mellitus, both animals and humans. The precise mechanisms responsible for these defects remain to be determined.

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