Phytohemagglutinin Transformation and Circulating Lymphocyte Subpopulations in Insulin-dependent Diabetic Patients

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SUMMARY

The lymphocyte transformation response to the mitogen phytohemagglutinin (PHA) was determined in forty well controlled insulin-dependent diabetics, forty matched normal subjects and fourteen poorly controlled insulin-dependent diabetics. There was no significant difference in the PHA responses of normal subjects and well controlled diabetics, but poorly controlled diabetics showed a marked depression of lymphocyte transformation.

Peripheral blood T and B lymphocyte subpopulations were also measured in fifteen normal subjects, fifteen well controlled diabetics and ten poorly controlled diabetics. The results showed no significant difference between normal and diabetic subjects, whether well or poorly controlled.

The depressed PHA response in poorly controlled diabetics would seem to reflect inadequately corrected metabolic disturbance rather than an inherent, genetically determined immunologic abnormality. DIABETES *23*:708-12, August, 1974.

Recent studies have examined the role of cell-mediated immune mechanisms in diabetes mellitus. For example, the leucocyte migration test (LMT)[1] has reportedly shown cellular hypersensitivity in diabetics against both nonspecific antigens (human and rat liver mitochondria[2]) and an antigen derived from the microsomal fraction of the islets of Langerhans (porcine pancreatic antigen[3]). In the latter study a positive correlation was found between the LMT response and delayed-type skin hypersensitivity to the antigen.[3]

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Other investigators have used the lymphocyte transformation response to the mitogen phytohemagglutinin (PHA)[4] as an in vitro test of cell-mediated immune function: some record the PHA response to be impaired with diabetic lymphocytes,[5] suggesting immunologic abnormality, while others report it to be normal.[6] To re-examine these results we have measured the lymphocyte transformation response to PHA in well controlled and poorly controlled insulin-dependent diabetics and in normal subjects; in addition erythrocyte rosette [7-9] and in indirect immunofluorescence [7,9] technics have been used to compare the numbers of circulating T and B lymphocytes in peripheral blood from these three groups.

PATIENTS AND METHODS

Patients

PHA responses were studied in forty well controlled diabetic and forty normal subjects who were carefully matched for age and sex. The diabetic subjects (twenty-two women, eighteen men; mean age 42.4 years) were insulin-dependent, attended an outpatient clinic, and were free from infection on the day of study. The control subjects were healthy volunteers, mainly laboratory personnel or hospital outpatients not known to have endocrine disease or immunologic abnormality.

PHA responses were also studied in a group of fourteen poorly controlled insulin-dependent diabetics. These patients had been brought to an outpatient clinic for routine or emergency review and their disorder was judged to be poorly controlled by the following criteria: midmorning blood glucose exceeding 350

mg./100 ml.; recent increase in insulin requirements; the presence of heavy glycosuria and/or ketonuria. Some were subsequently admitted to the hospital for correction of their metabolic abnormalities but none had received antibiotics or drugs other than insulin at the time of study. Their clinical details are presented in table 1.

T and B cell subpopulations were measured in fifteen of each of the well controlled diabetic and normal subjects and in ten of the poorly controlled diabetic subjects.

Collection of blood samples and separation of lymphocytes

Venous blood was withdrawn at midmorning and was anticoagulated with preservative-free heparin (Weddell Pharmaceuticals or Evans Medical). A portion of each sample was used for measurement of total and differential white blood cell counts. Lymphocytes were separated from the remainder by density centrifugation on a Ficoll-Triosil gradient[10] and washed three times in Eagles' Basal Medium (EBM, Wellcome Reagents Ltd.). The cells were then resuspended in EBM with 10 per cent fetal calf serum (Wellcome), counted and the concentration adjusted to 1 x 10^6 cells per milliliter. All cell suspensions contained more than 95 per cent lymphocytes with a viability greater than 98 per cent on trypan blue exclusion. Lymphocytes from the same sample were used for the PHA test and for T and B cell estimations.

Lymphocyte culture with PHA

Stock PHA (PHA-P, Difco) was diluted in EBM with 10 per cent fetal calf serum to give three solutions containing respectively 0.32, 0.63 and 1.25 μl. PHA per milliliter. Aliquots of 20 μl. of each solution were pipetted into the wells of Cooke Microtiter trays (Flow Laboratories) and 2 x 10^5 cells added to each well. All determinations were performed in triplicate and included control cultures without PHA. The microtrays were gassed with an air-5 per cent CO_2 mixture and incubated at 37° C. in sealed containers.

^{3}H-thymidine of specific activity 5 Ci./mmol (Radiochemical Centre, Amersham) was diluted with sterile saline to give a working concentration of 2 μCi./100 μl. Twenty microliters of this solution (i.e. 0.4 μCi. ^{3}H-thymidine) was added to each culture after forty-four hours' incubation. The cultures were regassed and reincubated for four hours. The contents of the wells were then pipetted on to fiberglass filter papers (Whatman GF/C) which were air-dried, washed successively with cold 5 per cent trichloracetic acid, phosphate buffered saline and absolute methanol, and finally placed in Packard glass counting vials. Five milliliters of scintillation fluid (NE233, Nuclear Enterprises Ltd.) was added to each vial and the samples counted for sixty seconds in an automatic beta counter (Packard 2425), the results being expressed as counts per minute (cpm).

Identification of T lymphocytes by sheep erythrocyte rosettes

The technic used[7] to identify E-rosettes was derived from that of Jondal et al.[8] and incorporated the modifications of Stjernsward et al.[9]

Identification of B lymphocytes

Two technics were used: a rosette technic[8,9] using sheep red cells coated with antibody and complement (EAC rosettes), and indirect immunofluorescence,[7,8] whereby the cells are distinguished by surface immunoglobulin marker.

TABLE 1

Clinical details of the fourteen poorly controlled diabetic patients in whom studies were undertaken

Age/ Sex	Duration of Diabetes (yrs.)	Blood glucose (mg. per cent)	Cause of poor control
24/F	10.0	358	Depressive illness
37/F	0.3	355	Recent diagnosis. Establishing control
48/F	5.2	450	Urinary tract infection
60/F	16.0	355	Emotional stress
61/F	11.0	352	Upper respiratory tract infection
66/F	5.0	440	Foot infection
68/F	0.05	385	New diagnosis. Establishing control
69/F	7.1	650	Congestive cardiac failure
17/F	6.9	510	Ketoacidosis, cause unknown
19/M	10.0	365	Skin sepsis, chest infection
28/M	13.0	408	Alcoholic, drinking bout
45/M	6.0	400	Acute bronchitis
46/M	9.0	360	Dental infection
60/M	3.7	800	Bronchopneumonia

RESULTS

Lymphocyte transformation with PHA

The dose-response curves of lymphocytes from well controlled diabetic and normal subjects are shown in figure 1. The mean transformation responses in the diabetics to the three doses of PHA employed were 17.0 ± 4.3, 20.8 ± 4.2 and 21.4 ± 4.3, respectively (expressed as cpm x 10^3 ± S.E.M.). The corresponding values in the controls were 15.3 ± 4.3, 19.7 ± 3.9 and 21.8 ± 3.9. Both curves are virtually identical and the lymphocyte transformation response to PHA is not abnormal in well controlled diabetics.

The dose-response curve of lymphocytes from the fourteen poorly controlled diabetics is compared in

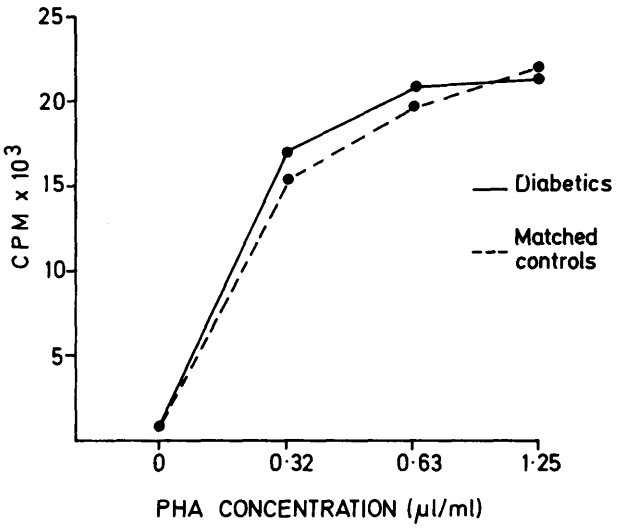

FIG. 1. Mean lymphocyte transformation responses in forty well controlled insulin-dependent diabetic (———) and forty age- and sex-matched normal subjects (-----) at three concentrations of PHA. The two dose-response curves do not differ significantly. S.E.M.'s are omitted for clarity but are given in the text.

figure 2 with the curve obtained from fourteen of the well controlled diabetics, matched as closely as possible for age, sex and duration of diabetes. The mean transformation responses in poorly controlled diabetics to the three doses of PHA were 9.0 ± 1.33, 9.5 ± 1.4 and 8.2 ± 0.6, respectively. The corresponding values in well controlled diabetics were 16.5 ± 2.0, 20.2 ± 1.9 and 21.3 ± 2.0. These results show a statistically significant reduction of PHA response in poorly controlled insulin-dependent diabetics at all three concentrations of PHA ($p<0.001$ by the Student *t* test).

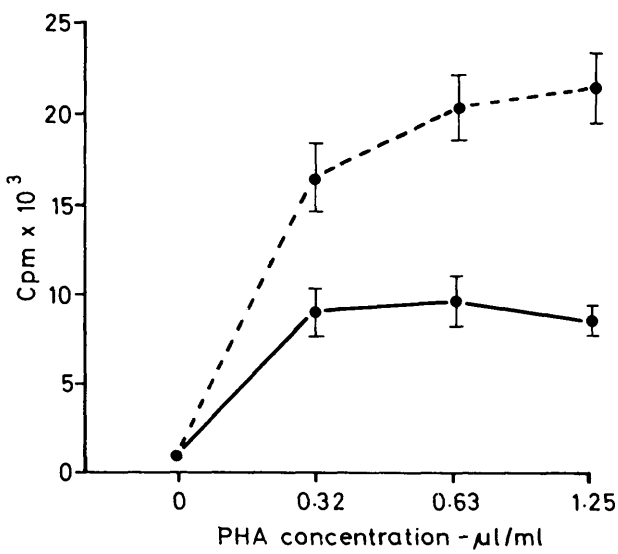

FIG. 2. Lymphocyte transformation responses (mean ± S.E.M.) in fourteen poorly controlled insulin-dependent diabetics (——) and fourteen well controlled insulin-dependent diabetics (----), who were matched for age, sex and duration of diabetes. The mean response in the poorly controlled diabetics is significantly lower ($p < 0.001$) at all concentrations of PHA.

Populations of T and B lymphocytes in peripheral blood

Tables 2, 3 and 4 list the total lymphocyte counts and the numbers of circulating T and B cells, expressed both as absolutes and percentages of total lymphocyte counts, in samples taken respectively from fifteen well controlled diabetics, fifteen normal subjects and ten poorly controlled diabetics. The results show no significant difference in the mean number and percentage of T cells (assessed by E-rosettes) or B cells (assessed by EAC rosettes or immunofluorescence technics). Circulating lymphocyte subpopulations are therefore normal in diabetics when obtained at times of 'good' and 'poor' metabolic control.

DISCUSSION

The above results confirm that lymphocyte transformation to blast cells by PHA, generally accepted as an in vitro test of cell-mediated immunologic response,[11,12] is normal in well controlled insulin-dependent diabetics when compared with matched normal controls. This agrees with the results of Ragab et al.[6] who found no differences in the PHA responses of lymphocytes from twenty-three diabetics and twenty-four controls. Contrary results (depression of PHA response) reported by Brody and Merlie[5] were based on the observations in six elderly diabetics who had persistent glycosuria and marked hyperglycemia (blood glucose 300 to 514 mg. per 100 ml.), and had not taken insulin between twelve and twenty-four hours before the lymphocytes were obtained for study. It therefore appears likely that these results reflect the metabolic disturbance in poorly controlled diabetes, rather than any inherent immunologic abnormality, and the present findings in poorly controlled diabetic subjects agree with this hypothesis.

Further support for the view that depressed PHA response in diabetics is due to metabolic abnormality is provided by the finding of normal numbers of circulating T and B lymphocytes in diabetics, irrespective of whether they were well or poorly controlled at the time of study. Since PHA response is considered mainly to test the function of the T-cell population,[11] it would be surprising to find a depression of PHA response in subjects with normal numbers of T cells;

TABLE 2

Total lymphocyte counts and subpopulations of T and B lymphocytes in peripheral blood from fifteen well controlled insulin-dependent diabetic patients. (E) = T lymphocytes identified by sheep erythrocyte rosettes. (EAC) = B lymphocytes identified by erythrocyte-antibody-complement rosettes. (IF) = B lymphocytes identified by indirect immunofluorescence.

Age/Sex	Total Lymphocyte count/mm³	T-cells (E) number	T-cells (E) per cent	B-cells (EAC) number	B-cells (EAC) per cent	B-cells (IF) number	B-cells (IF) per cent
22/F	1,403	878	62.6	449	32.0	—	—
28/F	2,350	1,465	62.3	—	—	357	15.2
35/F	1,566	1,038	66.3	188	12.0	227	14.5
55/F	2,560	1,318	51.5	—	—	294	11.5
56/F	2,211	1,282	58.0	104	4.7	736	33.3
59/F	1,320	854	64.7	121	9.2	300	22.7
62/F	671	374	55.8	203	30.3	221	33.0
62/F	1,360	743	54.6	—	—	299	22.0
68/F	2,132	1,552	72.8	544	25.5	452	21.2
17/M	2,464	1,627	65.9	315	12.8	490	19.9
19/M	3,250	1,570	48.3	504	15.5	523	16.1
36/M	1,975	1,284	65.0	375	19.0	257	13.0
42/M	1,682	1,182	70.3	579	34.4	—	—
63/M	1,440	935	64.9	—	—	2,321	16.1
Mean	1,879	1,153	62.0	338	19.5	375	20.4
±S.E.M.	165	91	1.79	56	3.29	42	1.98

except perhaps in diseases such as breast cancer,[13] multiple sclerosis,[14] or active syphilis[15] where the serum itself may contain factors inhibiting lymphocyte transformation, and in subjects treated with the drug co-trimoxazole.[16] The possibility of finding inhibitory factors in the sera of poorly controlled diabetics is presently being investigated and preliminary results suggest that hyperglycemia per se may contribute to depressed lymphocyte transformation in these patients. Finally, it should be noted that the demonstration of a normal PHA response and T-cell population in well controlled insulin-dependent diabetics does not exclude the possibility that smaller numbers of circulating lymphocytes in such patients may show transformation when exposed to a specific antigen, perhaps derived from pancreatic islet tissue.

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TABLE 3

Total lymphocyte counts and subpopulations of T and B lymphocytes in peripheral blood from fifteen normal subjects. Abbreviations as for table 2.

Age/Sex	Total Lymphocyte count/mm³	T-cells (E) number	T-cells (E) per cent	B-cells (EAC) number	B-cells (EAC) per cent	B-cells (IF) number	B-cells (IF) per cent
27/F	1,333	857	64.3	216	16.2	—	—
27/F	710	474	66.8	192	27.0	90	12.7
38/F	1,976	1,320	66.8	174	8.8	326	16.5
47/F	1,606	896	55.8	527	32.8	390	24.3
55/F	1,817	950	52.3	136	7.5	—	—
57/F	770	506	65.7	218	28.3	123	16.0
57/F	2,667	1,560	58.5	494	18.5	416	15.6
64/F	2,184	1,223	56.0	109	5.0	618	28.3
69/F	2,054	1,516	73.8	496	24.1	251	12.2
18/M	3,000	2,223	74.1	1,125	37.5	690	23.0
25/M	700	513	73.3	86	12.3	123	17.5
35/M	2,379	1,421	59.3	43	1.8	816	34.3
42/M	1,974	1,137	58.4	516	26.5	405	20.8
66/M	1,672	1,167	69.8	274	16.4	—	—
Mean	1,855	1,194	64.4	323	19.4	415	20.5
± S.E.M.	194	112	1.64	73	4.02	60	2.56

TABLE 4

Total lymphocyte counts and subpopulations of T and B lymphocytes in peripheral blood from ten poorly controlled insulin-dependent diabetic patients. Abbreviations as for table 2.

Age/Sex	Total Lymphocyte count/mm³	T-cells (E) number	T-cells (E) per cent	B-cells (IF) number	B-cells (IF) per cent
24/F	1,035	735	71.0	207	20.0
37/F	2,700	2,173	80.5	259	9.6
60/F	1,936	1,073	55.4	407	21.0
61/F	2,430	1,638	67.4	676	27.8
66/F	2,436	1,571	64.5	470	19.3
68/F	1,850	1,166	63.0	487	26.3
19/M	600	303	50.5	102	17.0
28/M	1,472	883	60.0	202	13.7
45/M	1,971	1,242	63.0	242	12.3
46/M	1,950	1,225	62.8	558	28.6
Mean	1,838	1,201	63.8	361	19.5
± S.E.M.	145	109	1.59	38	3.25

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