Clin. exp. Immunol. (1974) **18,** 461–467.

CELL-MEDIATED IMMUNITY IN DIABETES MELLITUS*

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(*Received* 4 *March* 1974)

SUMMARY

The quantified lymphocyte response to phytohaemagglutin (PHA) was investigated in fifty-six control subjects, classed according to age and sex, in twenty-nine insulin-dependent (IDD) and eight non-insulin-dependent diabetics. In nine cases one-way mixed lymphocyte cultures (MLC) were performed using mixed lymphocytes from IDD and paired controls. In the controls, a reduction of the response to PHA was observed with ageing; this was more marked in women than in men. The diabetics gave a greatly impaired response to PHA but only a slightly depressed response in MLC. These anomalies are not influenced by age but by the severity of the diabetes.

INTRODUCTION

The susceptibility of diabetics to tuberculosis, mycosis and staphyloccoci is well documented (Eisert, 1965; Joslin *et al.*, 1959). It is highest in poorly controlled patients (Derot, 1962). The susceptibility to *Staphyloccocus* infections has been ascribed to impaired leucocyte function (Mowat & Baum, 1971; Perillie, Nolan & Finch, 1962; Rohmann, 1966). An impairment of cell-mediated immunity (CMI) would account for the higher incidence and severity of tuberculosis or mycotic infections (Mackaness, 1971). So far, two studies only with different results have been published on this subject (Brody & Merlie, 1970; Ragab, Hazlett & Cowan, 1972).

In the present work, CMI was assessed by a quantified lymphocyte response to phytohaemagglutinin (PHA) and to allogenic cells in one-way mixed lymphocyte culture (MLC). Both reactions are considered as *in vitro* tests of CMI (Fudenberg *et al.*, 1971; Bach & Voynow, 1966), although their biological significance is different. PHA, a non-specific mitogen, activates T lymphocytes through receptors which are distinct from antigen receptors, whereas the lymphocyte transformation observed in MLC results from an *in vitro* primary immune reaction mainly involving T lymphocytes (Revillard, 1971; Schwarz & Robson, 1971).

* Part of this work was presented in a lecture at the Academie Royale de Médecine de Belgique, 1972.

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MATERIALS AND METHODS

Subjects tested

Twenty-nine insulin-dependent diabetics (IDD) were tested with PHA (fifteen women and fourteen men). Their ages ranged from 17 to 75 years and the duration of the diabetes from 1 to 27 years. At the time of testing, ten patients were under poor control, six had ketone bodies in the urine and two others were severely infected. Nine other IDD were tested for MLC (ages ranging from 17 to 69 years; seven men and two women). None of these was in acidosis when tested. The PHA test was also performed in eight non-insulin-dependent diabetics (NIDD) (five men and three women; ages ranging from 55 to 80 years) and in fifty-six control subjects. Forty-four of the latter were healthy blood donors and twelve were hospitalized patients without diseases or therapy liable to alter their immunological status. Their diagnoses were cerebrovascular accident, psychic diseases, pulmonary emphysema and chronic heart disease.

Lymphocyte cultures

The method was adapted from Sengar & Terasaki (1971) with the following modifications. The blood was defibrinated on glass beads and the lymphocytes were separated on Ficoll–Isopaque gradient. Aliquots of 2×10^5 lymphocytes in 0·2 ml of culture medium were introduced into polyethylene microtubes (Beckman, 0·4 ml).

These were capped and incubated at 37°C in a 5% CO_2 enriched air atmosphere. The culture medium consisted of MEM (Eagle's minimal essential medium for tissue culture), glutamine (1 mM/ml) and 20% human AB serum. The pH was adjusted to 7·3 with natrium bicarbonate. Batches of AB serum and of PHA (Wellcome, batch number K4402) were pooled and used throughout the whole work. Each culture was performed five times. Each test consisted of at least one control culture (lymphocytes in medium alone) and one culture with PHA at a final concentration of 1 μg/ml. After 3 days at 37°C, 1 μCi (0·01 ml) of tritiated thymidine (Centre Nucléaire de Mol; specific activity 2 Ci/mM) was added to each tube and mixed. The cultures were harvested after an additional overnight (14–16 hr) incubation. Harvesting and isotope counting were done according to Sengar and Terasaki. Results were expressed as counts per 10 min per microtube; no correction was made for the quenching.

Standardization of the quantified PHA test

A dose–response curve was first established (Fig. 1) to select the most discriminative concentration of PHA which should be infraoptimal (Hosking, Fitzgerald & Simons, 1971; Oppenheim, Blaese & Waldmann, 1970).

The PHA concentration of 1 μg/ml was selected because it yields the best reproducible results, and is sufficiently discriminative. The reproducibility was tested at monthly intervals on two healthy young men (variation coefficient lower than 30%, Table 1).

Mixed lymphocytes cultures

One-way stimulation was achieved by mitomycin C treatment at a concentration of 30 μg/ml.

Each patient (D) was tested against an unrelated healthy control (C) of the same age and sex. Two sets (D+Cm; C+Dm) of five replicates containing 10^5 responding lymphocytes

(D or C) and 10^5 mitomycin-treated cells (Dm or Cm) in 0·2 ml of culture medium, were done for each patient. The cultures were harvested after 7 days of incubation.

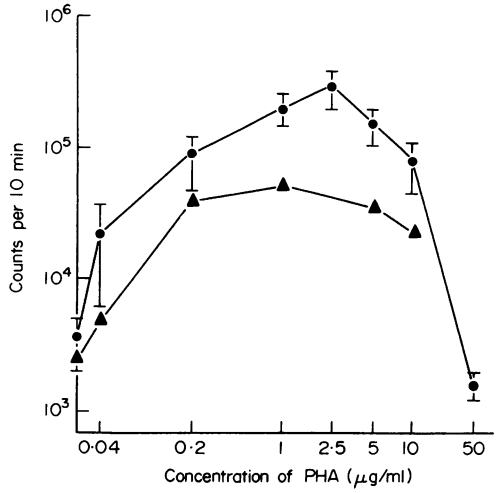

FIG. 1. Effect of various concentrations of PHA on lymphocyte cultures: mean of six normal subjects (●) and of one insulin-dependent diabetic (▲).

TABLE 1. Reproducibility of the response to PHA

Test number	Counts per 10 min ± s.d. in five replicates	
	Subject 1	Subject 2
1	163·562 ± 43·015	181·558 ± 19·306
2	219·475 ± 41·496	152·852 ± 13·695
3	141·178 ± 34·741	205·000 ± 19·600
4	157·775 ± 11·934	172·275 ± 18·275
5		107·375 ± 10·400
Mean results	172·288 ± 42·204	164·730 ± 37·941
Variation with time	24·5%	23·03%

Statistical analyses

The variation coefficient (V) was calculated for each set of five replicates. When V was greater than 30%, the test was discarded. Non-parametric test of Mann–Whitney–Wilcoxon were used to compare the different populations.

RESULTS

The PHA response of the controls are strikingly influenced by age and sex (Fig. 2). Under the age of forty the response is the same in men and in women, except when the latter are preg-

nant or on oral contraceptives (Table 2). In both sexes, there is a decreased response with ageing, this phenomenon being more marked in women.

Insulin-dependent diabetics show an abnormally low response to PHA when compared with their matched controls ($P<0{\cdot}01$). Moreover, this is not influenced by age or sex. The response of NIDD is depressed but much less so than IDD.

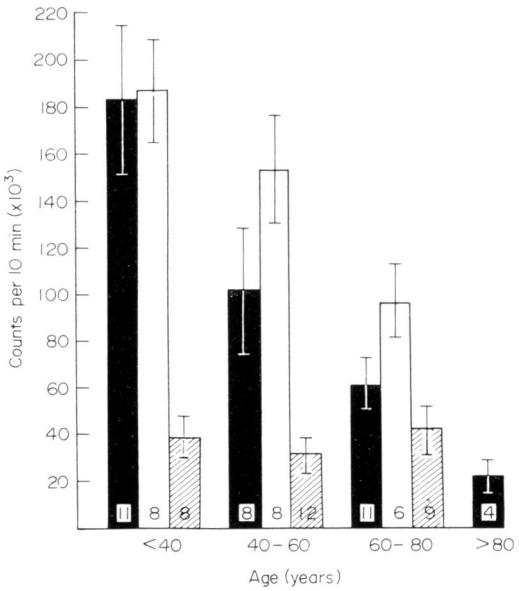

FIG. 2. Lymphocyte transformation with PHA in normal subjects and insulin-dependent diabetics. The [^{3}H]thymidine incorporation, expressed as counts per 10 min, corresponds to 2×10^5 lymphocytes incubated for 3 days with PHA (1 μg/ml), average values of plotted results and limits of confidence ($P = 0{\cdot}05$) are represented. The number of patients tested is indicated at the base of each column. None of the female patients or controls are pregnant or on oral contraceptives. Solid columns = female controls. Open columns = male controls. Hatched columns = diabetics.

TABLE 2. Influence of oral contraceptives or pregnancy on the PHA test

	Women taking oral contraceptives (five subjects)	Pregnant women (three subjects)	Controls* (eight subjects)
Counts per 10 min ± s.d. (mean value of plotted results)	55·540 ± 11·671	46·338 ± 5·837	170·892 ± 39·146

* The controls consist of selected subjects closely matched for age.

The response of diabetic lymphocytes in one-way MLC (D+Cm) is depressed in five cases; however, the mean result of the nine studied patients is not significantly lower than observed in their paired controls (C+Dm) (Table 3).

It is to be noted that the lymphocyte response after 7 days of incubation with PHA (instead of 3 days) is more strongly decreased in the normal subjects than in the diabetics.

TABLE 3. One-way mixed lymphocyte cultures in diabetics and matched controls

Age and sex of the paried subjects	[^{3}H]Thymidine incorporation (counts per 10 min ± s.d.)	
	D+Cm (Lymphocytes of controls blocked against lymphocytes of diabetics)	C+Dm (Lymphocytes of diabetics blocked against lymphocytes of controls)
17 M	37·978 ± 6·997	56·609 ± 3·642
30 M	20·470 ± 6·280	26·330 ± 3·070
36 M	38·150 ± 5·480	39·600 ± 6·910
49 M	39·748 ± 6·983	43·020 ± 8·624
52 M	46·552 ± 6·769	37·389 ± 6·20
58 M	38·211 ± 7·715	32·380 ± 7·342
69 M	29·585 ± 8·431	37·341 ± 11·534
35 F	17·661 ± 3·820	22·586 ± 0·045
65 F	16·353 ± 5·426	35·263 ± 12·145
Mean results	32·446 ± 10·550	35·912 ± 10·706

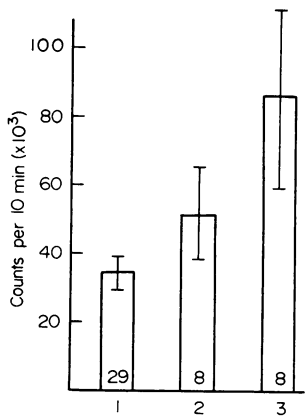

FIG. 3. PHA response of insulin-dependent and non-insulin-dependent diabetics. (1) Insulin-treated diabetics. (2) Non-insulin-dependent diabetics. (3) Matched controls of (2). Average values and limits of confidence ($P = 0{\cdot}05$). The number of subjects tested is indicated at the base of each column. The difference between (2) and (3) is significant ($P = 0{\cdot}05$; Man–Whitney–Wilcoxon non-parametric test).

DISCUSSION

The stimulation of human peripheral lymphocytes by PHA is considered to test the overall functional status of thymus-dependent cells (Fudenberg *et al.*, 1971).

The quantification and standardization by the method here described yields a satisfactory

reproductibility allowing its clinical applications (Richter & Naspitz, 1967; Pentycross, 1969).

This study confirms the existence of an impaired response to PHA in old people (Pisciotta *et al.*, 1967), in women taking oral contraceptives (Hagen & Froland, 1972) or pregnant women (Purtillo, Hallgren & Yunis, 1972). It further shows a drop in women after the menopause which contrast unexpectedly with the slow age reduction in men.

The main finding of this work is the greatly altered response to PHA in insulin-dependent diabetics. This defect is influenced by the severity of the diabetes. Indeed, NIDD have a significantly better response to PHA than IDD and amongst the latter the lowest lymphocytes stimulations are observed in poorly controlled patients (Table 4).

TABLE 4. Comparison of the lymphocyte response of well controlled and poorly controlled diabetics

	Well controlled when tested*	Poor controlled when tested†
Number of subjects	19	10
Mean (counts per 10 min)	40·261	28·563
S.d.	±21·188	±12·682

* Fasting glycaemia <150 mg%; no glycosuria, no hypoglycaemia.
† Fasting glycaemia >250 mg%; six were in ketoacidosis.

The impaired response to PHA is not due to a poor survival of the diabetic lymphocytes in the cultures as shown by the [^{3}H]thymidine incorporation in the non-stimulated cultures (2800 counts per 10 min/3300 counts per 10 min in normal subjects) and after 7 days of incubation with PHA. A metabolic disturbance related to a relative insulin deficiency is also unlikely, as the addition of 0·2 u of insulin per culture did not influence the response of five IDD compared to five controls and three NIDD.

Another finding of this study is the contrast between the lymphocyte responses to PHA and in MLC. The same dissociation has been reported in patients after thoracic duct drainage (Revillard & Brochier, 1971), in old age (Heine *et al.*, 1971) and in some cases of thymic malformation (Meuwissen *et al.*, 1968). In all these cases, there is a depletion of circulating T lymphocytes. Such a defect could explain the data here presented and the observation made by others (Ragab *et al.*, 1972) that diabetic subjects still have a normal lymphocytic response to specific antigens like candidin. Preliminary results of Hubert *et al.* (1974) indicate a moderate but significant reduction of the circulating B cells in diabetics.

Another possibility would be the inactivation of the lymphocyte receptors to PHA. Indeed, treatment of lymphocytes with neuraminidase, which blocks the PHA receptors, leads to an impaired response of these cells to PHA without altering their response in MLC (Lindahl-Kiessling & Peterson, 1969).

Obviously, these and other possible explanations remain to be tested by further work.

ACKNOWLEDGMENTS

The authors acknowledge Mrs Branders and Mrs Noël for their skilful help.

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