Effect of phytohaemagglutinin in the leucocyte migration inhibition test as a measure of cell-mediated immunity

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SYNOPSIS Evaluation of responsiveness to phytohaemagglutinin (PHA) in the leucocyte-migration inhibition test has been extended to a greater number of patients and control subjects to explore the range of the test. The patients, mainly with sarcoidosis, were found to be less responsive than controls. The conditions of the test in terms of concentration of PHA, duration of culture, and serum concentration used were investigated and standards suggested. Intraperson variation and the influence of age were also evaluated.

Lymphocyte response to PHA stimulation in culture has been used for several years as a guide *in vitro* to cell-mediated immunity *in vivo*, but it has remained largely a research tool. It has recently been reported that PHA produces inhibition of leucocyte migration under the conditions of the leucocyte-migration inhibition test and that the results appear to correlate with cell-mediated immunity *in vivo* (Morison, 1973). There appears to be a need for a simple and practical test of lymphocyte function and the leucocyte migration inhibition test would hold considerable advantages over lymphocyte transformation as it is easier to perform, requires less equipment, and gives a result within 24 hours. However, before it can be accepted as an alternative the scope of the test must be explored and standard conditions established.

The aim of the present study was first, to extend the number of patients and controls examined, and secondly to investigate some of the variables which might influence the results. The dose of PHA, the duration of the test, and culture conditions were examined.

Patients and Method

PATIENTS

A total of 16 patients were examined. Fourteen had sarcoidosis, one had chronic lymphatic leukaemia, and one had Hodgkin's disease. The age range was 22-72 years, with a mean age of 42 years.

CONTROLS

Sixteen healthy control subjects were examined, matched for age and sex to the patients. The age range was 24-74 years, with a mean age of 43 years.

LEUCOCYTE MIGRATION INHIBITION

This test was performed essentially according to the technique of Søbørg and Bendixen (Søbørg and Bendixen, 1967; Søbørg, 1971). Thirty ml venous blood is collected in an heparinized syringe, transferred to 10 ml plastic tubes, and allowed to sediment spontaneously for one hour at 37°C. The plasma supernatant is aspirated to within 5 mm of the red cell layer and centrifuged at $225 \times g$ for five minutes. The plasma is aspirated, the white cells resuspended in 5 ml Hank's balanced salt solution, and washed three times in this solution. The cell population consists of 20-40% mononuclear cells and 60-80% granulocytes. After the washing procedure the cells are resuspended in tissue culture medium 199 (Burroughs Wellcome) with 10% inactivated horse serum added and drawn into 75 mm $\times$ 1 mm capillary tubes, the ends of which are sealed using Cristaseal (Hawksley). Usually 15 tubes are obtained from a 30 ml blood sample. The capillary tubes are centrifuged at $225 \times g$ for 10 minutes and each tube is cut 0.5 mm below the cell-fluid interface. Immediately, the cell-containing part of the tube is placed in a culture chamber of 0.5 ml capacity (Sterilin microchambers) fixed in position using silicone wax and the chamber filled with tissue culture medium 199 plus 10% horse serum. The PHA (Burroughs Wellcome, freeze dried grade) is

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added to activated chambers and an equal volume of normal saline to control chambers. A cover slip is placed on top and pressed so that it is held by surface tension and the chambers are then placed in a humidified atmosphere at 37°C. At the end of the incubation period the area of migration is mapped using a projection microscope (plan 2.5 objective) and measured by planimetry. The migration index is defined as the ratio between the area of migration in the presence of PHA and the area of migration in control chambers. The value of the normal migration index is therefore 1.0, values less than 1.0 indicating inhibition of migration.

A sterile procedure was carried through as far as possible and random sampling of chambers has not shown any bacterial growth. Glassware was not siliconized as it was shown not to affect the results. The diameter of the migration area of the cells in non-activated chambers varies between individuals, ranging from 5 to 10 mm and this variation is not related to whether the subjects being examined are patients or controls. It depends, no doubt, on the number of cells in the capillary tubes and on the cell population. However, by using a migration index this variation is eliminated. In the present study all cultures were set up in triplicate and results only

included when the difference between chambers was less than $\pm 10\%$. This limit was seldom exceeded.

Results and Discussion

PHA CONCENTRATION

A number of concentrations were tried ranging from 0.1 $\mu\text{g/ml}$ to 100 $\mu\text{g/ml}$. The results are shown in fig 1 which demonstrates good separation between most patients and control subjects at the lower concentrations of 0.1 to 10 $\mu\text{g/ml}$, but considerable overlap at concentration of 50 to 100 $\mu\text{g/ml}$. In fig 2 a curve for the mean of the results in both patients and controls indicates that greatest separation is given by a concentration of 5 $\mu\text{g/ml}$. For all subsequent experiments introducing other variables this was the standard concentration used.

DURATION OF CULTURE

A culture time of 18 to 24 hours has usually been reported as adequate for the leucocyte migration test when antigen is used to activate the cultures. The influence of time on the result when using PHA in the system was assessed and the results are shown in figure 3. It can be seen that even at six hours a result can be obtained and that time possibly has

Fig 1 Response to increasing PHA concentrations in patients and control subjects.

Fig 2 Mean response curves to increasing PHA concentrations in patients and control subjects.

more influence on patients with defective lymphocyte function than on normal controls, there being a gradual increase in responsiveness in the patients.

CONDITIONS OF THE TEST

Ten per cent horse serum was added to the culture medium in the leucocyte migration inhibition test. The effect of increasing that concentration is shown in table I. In the first two columns, control chambers containing 25 and 50% horse serum have been compared with controls containing only 10% and the resultant index indicates a marked reduction in migration area as serum concentration rises. In the

Fig 3 Response obtained at six hourly intervals to 5 µg/ml PHA.

last three columns the migration indices for each serum concentration are given and in most cases there is a reduction of the index as serum concentration rises. However, it was found that at the higher serum concentrations the cells were clumped and not evenly spread, this effect being more marked at 50% compared with 25% horse serum. This observation, coupled with a reduction in accuracy as the area of migration falls, would indicate that 10% is the most suitable concentration of horse serum for the system.

After the white cells are separated from the blood they are washed in Hank's BSS to remove traces of serum. Three washes are usually considered adequate but as this possibly does not remove all traces of serum, six washes were tried on two patients and two control subjects. The area of migration in all chambers was found to be greatly reduced, the effect on the patients (90% reduction) being more marked than in the control subjects (30% reduction). It is felt that such a reduction decreases the accuracy of the test and, therefore, three washes are preferable.

	Control Cultures with PHA (5 µg/ml) in Addition of				
	25%HS	50%HS	10%HS	25%HS	50%HS
Patient 1	0.53	0.42	0.42	0.47	0.39
Patient 2	0.88	0.77	1.00	0.76	0.65
Control subject 1	0.54	0.31	0.16	0.14	0.22
Control subject 2	0.74	0.61	0.18	0.14	0.04
Control subject 3	0.76	0.53	0.23	0.16	0.15

Table I Effect of increasing horse serum concentration to 25 and 50%¹

¹Indices in first two columns are comparisons with 10% control cultures. Other columns show effect of adding 5 µg/ml PHA, the controls in these cases containing the same concentration of horse serum (HS).

It has previously been reported that the interval between collecting the blood and completion of the test is critical in the standard leucocyte migration inhibition test (Søberg, 1971). During the present study it was similarly found that the test should ideally be set up within two hours from the time of venepuncture and three hours is the maximum interval for a worthwhile result. Further delay results in reduction of the areas of migration, presumably due to death of cells.

INTRAPERSON VARIATION OF RESPONSE

To assess the reproducibility of the test and the degree of intraperson variation, two patients and three control subjects were examined twice at intervals of two to four weeks. The results are shown in table II. Some variation is shown but this is felt to lie within the accuracy of the test system.

INFLUENCE OF AGE ON RESPONSE

Immunological competence may well vary with age and, therefore, the control subjects were matched for age to the patients in an attempt to eliminate this variable. To assess the influence of aging on the

	Migration Index 1	Migration Index 2
Patient 1	0.82	0.78
Patient 2	0.75	0.72
Control subject 1	0.14	0.02
Control subject 2	0.11	0.13
Control subject 3	0.04	0.09

Table II Response to 5 µg/ml PHA as measured on two occasions at 2 to 4 week intervals

response to PHA the results for the control subjects at a PHA concentration of 5 µg/ml has been graphed in fig 4 according to their age. There is no apparent difference between the groups and the mean index for the 20-30 year group of 0.14 is almost the same as that for the 60-80 year group of 0.15.

Conclusions

This study has evaluated some of the variables in the leucocyte migration inhibition test using PHA as a stimulant of lymphocyte function. The following conclusions regarding the technique of the test can

Fig 4 Response in 16 control subjects to 5 µg/ml PHA arranged according to age.

be drawn from the results: (1) PHA should be used in a dose of 5 µg/ml; (2) the test can be read at six hours or left overnight, for 18 to 24 hours; (3) 10% horse serum in the culture medium gives a better result than higher concentrations; (4) three washings of the cells are adequate.

Patients with sarcoidosis, chronic lymphatic leukaemia, and Hodgkin's disease were chosen as it is considered that they frequently have a cell-mediated immune deficiency. However, not all such patients would be expected to have the same degree of deficiency and this is demonstrated by the wide scatter of results. It is tentatively suggested from the information in fig 1 that a migration index of 0.5 or more, at a PHA concentration of 5 µg/ml indicates that the subjects' lymphocytes are abnormal, between 0.3 and 0.5 is equivocal, and below 0.2 is normal for this system.

Till now the leucocyte migration inhibition test has only been applied to evaluating antigenic response, but from this study it would appear that the use of a mitogen such as PHA can supply additional information about the immunological competence of an individual. Although the mechanism of action of PHA on lymphocytes is not fully understood its use in the lymphocyte transformation test is well established. However, use of PHA in the

leucocyte migration inhibition test holds three main advantages over its use in the lymphocyte transformation test: (1) it is relatively easier to perform requiring a minimum of technical expertise; (2) equipment is simple and inexpensive; and (3) a result is available as early as six hours from the time of setting up the test compared with two to three days for lymphocyte transformation. It is therefore suggested that the leucocyte migration inhibition test using PHA could be readily applied in routine laboratories as a test *in vitro* of patients' cell-mediated immunity *in vivo*.

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