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MIGRATION INHIBITION OF HUMAN LEUKOCYTES MIXED WITH PHYTOHEMAGGLUTININ-PREINCUBATED MONONUCLEAR LEUKOCYTES

By

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The proliferative response of human lymphocytes to phytohemagglutinin (PHA) has been used for several years as an *in vitro* parameter of the T lymphocyte function. But the concept that PHA primarily stimulates human T cells has only recently been confirmed by studies of purified T and B lymphocyte populations (4). Lymphocytes activated by PHA will, however, not only transform to blast cells, but also produce migration inhibitory factor (MIF) (7) and other lymphokines (3, 9).

Even if lymphocytes stimulated by antigen or polyclonal mitogens (e.g. PHA) normally will produce lymphokines as well as proliferate, there are situations in which only one of the responses can be demonstrated. In some cases of chronic mucocutaneous candidiasis, candida antigen can induce blast transformation, but not lymphokine production (11). On the other hand, lymphocytes are still able to produce lymphokines even if their ability to proliferate has been abolished by X-irradiation (6, 12) or by cytostatics (2, 5). This dichotomy may reflect that two different lymphocyte populations are involved, one able to produce lymphokines and another to proliferate (10). Therefore, when lymphocytes are stimulated *in vitro* by specific antigens or polyclonal mitogens, it is desirable

to measure not only the transformation response, but also the production of lymphokines (e.g. MIF).

A difficulty in devising a test able to demonstrate PHA-induced MIF production is that PHA itself can inhibit cell migration. Recently, Morison (8) has described such a leukocyte migration inhibition test. He added PHA to the tissue culture medium of chambers containing capillary tubes packed with human peripheral blood leukocytes. By using a low concentration of PHA, he was able to inhibit the migration of leukocytes from normal controls, but not that of leukocytes from patients with sarcoidosis. He suggested that this was caused by a deficient lymphocyte function in patients with sarcoidosis. However, PHA-induced migration inhibition demonstrated by adding PHA to a mixture of lymphocytes and granulocytes may primarily be caused by a direct effect of PHA on the migrating polymorphs.

The present paper describes a test in which no PHA was added to the migrating polymorphonuclear leukocytes, but only to purified mononuclear leukocytes. After a short incubation with PHA, the mononuclear cells were washed thoroughly and then mixed with non-separated leukocytes. The migration inhibition of the mixed cell population was considered to be caused by the PHA-stimulated lymphocytes producing granulocyte-MIF.

MATERIALS AND METHODS

Blood specimens were obtained from normal healthy adults or from patients without diseases known to influence cell-mediated immunity. Patients under treatment with glucocorticoids or cytostatics were not included in the study. The blood used for preparation of non-separated leukocytes and purified mononuclear leukocytes was taken simultaneously. The agarose migration technique devised by Clausen (1) was used to demonstrate the leukocyte migration inhibition reaction.

Preparation of agarose medium. A solution of 2 per cent agarose (Litex, Glostrup, Denmark) in distilled water was prepared every 2 weeks and stored at 5° C. Fresh agarose culture medium was prepared daily. The 2 per cent agarose gel was heated in boiling water. After solution, the agarose was cooled to 47° C in a water bath. For preparing 100 ml agarose medium, 50 ml 2 per cent

agarose solution were added to a mixture (prewarmed to 47° C) of 31 ml sterile distilled water, 9 ml ten-fold concentrated TC 199 (TC-medium 199, 10 X, Difco Laboratories, Detroit, Mich., U.S.A.), 10 ml horse serum (Statens Serum-institut, Copenhagen, Denmark), and 540 μ l 10 per cent sodium bicarbonate (TC-bicarbonate solution 10 per cent, Difco Laboratories). The 47° C agarose culture medium was sucked into a prewarmed 25-ml burette, and 5-ml volumes transferred to 48 $\times$ 8.5 mm disposable plastic Petri dishes (Millipore Filter Corp., Bedford, Mass., U.S.A.). After the gel had solidified, the dishes were incubated at 37° C in a humidified atmosphere of 2 per cent CO₂ in air. The bicarbonate/CO₂ buffer resulted in a pH between 7.20 and 7.40. When the leukocyte cultures were to be made, six to eight wells of 2.3 mm diameter were punched in the gel.

Preparation of non-separated leukocytes. Eight milliliters of venous blood were collected in 115 $\times$ 13 mm polystyrene tubes (Nuncion tubes, Nunc, Roskilde, Denmark) each containing 250 units heparin (Heparin Leo, 5000 units per ml, Leo, Copenhagen, Denmark) and 2 ml 5 per cent dextran 250 (Dextran 250, mol. wt. 250,000, Pharmacia, Uppsala, Sweden) in saline (0.9 per cent) solution. After sedimentation for $\frac{3}{4}$ to 1 h at 37° C, the leukocyte-rich plasma was withdrawn and centrifuged at 220 g for 5 min. The cells were washed twice in Hanks' balanced salt solution, then resuspended in 8 ml TC 199 (TC-medium 199, Difco Laboratories) with 10 per cent horse serum and incubated at 37° C until use (i.e. in about 1½ h).

Preparation of purified mononuclear leukocytes. Venous blood with 250 units heparin per 10 ml blood was diluted with an equal volume of Hanks' balanced salt solution. Seven milliliters of the diluted blood were layered carefully upon 3 ml of a mixture of 9.6 per cent sodium metrizoate and 5.6 per cent Ficoll (Lymphoprep, Nyegaard & Co., Oslo, Norway). After centrifugation at 400 g for 20 min at room temperature, the mononuclear leukocytes (composed of about 90 per cent lymphocytes and 10 per cent monocytes) in interface between the plasma and the lymphoprep were pipetted off, washed three times in Hanks' balanced salt solution and resuspended in TC 199 with 10 per cent horse serum to a cell concentration of 4×10^6 /ml.

PHA stimulation of purified mononuclear leukocytes. A solution of PHA was prepared by adding 5 ml sterile distilled water to freeze-dried PHA (Phytohemagglutinin, HA 15, 5 ml dried, Wellcome Reagents Limited, Beckenham, England). Twenty microliters of the PHA solution were added to 1 ml of the suspension of purified mononuclear leukocytes. The PHA-containing cell suspension and a similar one without PHA were incubated for 30 min at 37° C. Then the cells were sedimented by centrifugation at 220 g for 5 min and washed three times in Hanks' balanced salt solution.

Migration of non-separated leukocytes mixed with PHA-preincubated mononuclear leukocytes. The suspension of non-separated leukocytes (see above) was centrifuged at 220 g for 10 min, and the cells resuspended in TC 199 with 10 per cent horse serum to a cell concentration of 2.3×10^6 /ml.

After the last washing of the mononuclear leukocytes, the cells were spun down and resuspended in 90 μ l of the suspension of non-separated leukocytes (i.e. 4×10^6 PHA-preincubated or control mononuclear leukocytes were mixed with 21×10^6 non-separated leukocytes from the same subject). After incubation for 30 min at 37° C, portions of 7 μ l from each of the mixed cell suspensions were placed in wells in agarose medium. The agarose cultures were incubated at 37° C in a humidified atmosphere of 2 per cent CO₂ in air. After 20 h the cultures were studied under a microscope, and the area of migration calculated from the average of two perpendicular diameters. The bottom of the well was not included in the migration area. The PHA-induced migration inhibition was expressed as a migration index (MI) which indicated the ratio between the average area of six to eight cultures containing PHA-preincubated mononuclear leukocytes and that of the same number of control cultures.

Deviations from the standard procedure. In some experiments, the PHA test was made not only as described above, but also with some deviations: 1) The mononuclear leukocytes were preincubated with various doses of PHA. 2) A varying number of PHA-preincubated mononuclear leukocytes were mixed with the same number of non-separated leukocytes (i.e. 21×10^6 non-separated leukocytes). 3) The PHA-preincubated mononuclear leukocytes were washed and then heated at 56° C for 30 min before being mixed with non-separated leukocytes. 4) The mononuclear leukocytes were preincubated with PHA which had been heated at 56° C for 30 min.

RESULTS

Addition of PHA-preincubated mononuclear leukocytes to non-separated leukocytes resulted in a migration inhibition of the mixed cell population (Table 1). The mean MI of 40 experiments was 0.50 (s.d. 0.12). The average control migration area in these experiments was 41.8 (s.d. 14.5) mm². The effect of PHA heated at 56° C for 30 min was the same as the effect of non-heated PHA in 10 experiments.

The dose-effect relationship between PHA dose and migration inhibition is shown in Table 2. Preincubation of mononuclear leukocytes with increasing doses of PHA from 1.25 to 20 μ l/ml resulted in decreasing MI. But when the PHA dose was increased further, a maximum migration inhibition was obtained in subject nos. 1, 4, 7, 8, and 9 (i.e. two or more of the highest PHA doses resulted in about the same migration inhibition). Fig. 1 shows a typical dose-response curve.

Table 3 shows that if the content of PHA-preincubated

TABLE 1
*Migration Inhibition of Non-separated Leukocytes Mixed with
 PHA-Preincubated Mononuclear Leukocytes.*

Subject no.	Migration index	Subject no.	Migration index
1	0.76	21	0.51
2	0.69	22	0.50
3	0.66	23	0.49
4	0.66	24	0.49
5	0.66	25	0.48
6	0.64	26	0.47
7	0.60	27	0.45
8	0.59	28	0.45
9	0.58	29	0.45
10	0.58	30	0.45
11	0.58	31	0.43
12	0.58	32	0.42
13	0.57	33	0.38
14	0.56	34	0.37
15	0.56	35	0.35
16	0.54	36	0.33
17	0.52	37	0.33
18	0.52	38	0.29
19	0.52	39	0.27
20	0.51	40	0.23

mononuclear leukocytes in the mixed cell population was increased from 16 per cent (i.e. the standard percentage) to 32 per cent, the average MI decreased only 0.03. If the content was lowered from 16 to 8 per cent, the average MI increased only 0.02. None of these MI differences were statistically significant ($P > 0.10$, the Wilcoxon test for pair differences). If the content of PHA-preincubated mononuclear leukocytes was decreased from 16 to 4 per cent, the average MI increased 0.22. This increase was significant ($P < 0.025$).

No migration inhibition was induced if the PHA-preincubated mononuclear leukocytes were heated at 56° C for

TABLE 2

Dose-Effect Relationship Between PHA Dose and Migration Inhibition of Non-separated Leukocytes Mixed with PHA-Preincubated Mononuclear Leukocytes.

Subject no.*	Migration indices								
	PHA dose (μ l/ml)								
	1.25	2.5	5	10	20	40	80	160	320
1				1.01	0.76	0.65	0.42	0.40	0.39
2	1.00	1.00	1.00	0.80	0.69	0.54			
3		1.00	0.95	0.75	0.66	0.58	0.42		
4			0.97	0.68	0.66	0.57	0.51	0.44	0.42
7	0.96	0.90	0.88	0.73	0.60	0.59	0.58		
8			0.95	0.89	0.59	0.59	0.55	0.50	0.50
9		0.95	0.93	0.73	0.58	0.44	0.39	0.35	0.35
12				0.65	0.58				
15				0.65	0.56				
23				0.55	0.49				
27			0.77	0.59	0.45				
29				0.93	0.45				
33	1.08	0.98	0.68	0.43	0.38	0.31			
34	1.00	0.94	0.79	0.50	0.37				
38	0.95	0.87	0.70	0.46	0.29				
39				0.40	0.27				

* The subjects are numbered as in Table 1.

30 min (Table 4). The control migration areas of non-separated leukocytes mixed with heated mononuclear leukocytes did not differ from those of the corresponding non-separated leukocytes mixed with non-heated mononuclear leukocytes.

DISCUSSION

In the present PHA test, PHA was not added directly to the migrating polymorphs. However, even if the PHA-preincubated mononuclear leukocytes had been washed thoroughly, some PHA bound to receptors on the cell membranes was probably transferred to the non-separated leukocytes. There-

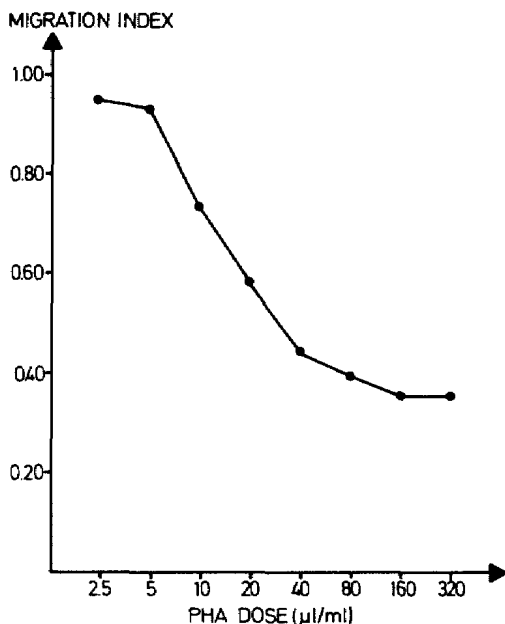


Fig. 1.

Dose-effect relationship between PHA dose and migration inhibition of human leukocytes. Purified mononuclear leukocytes were incubated with PHA for 30 min, then washed thoroughly and mixed with non-separated leukocytes which acted as migrating indicator cells. The migration inhibition was demonstrated by the agarose migration technique.

fore, PHA liberated from the preincubated cells or still bound to the cell membranes might have inhibited the migration of polymorphs directly. But heat-treated (56°C for 30 min) PHA-preincubated mononuclear leukocytes did not induce migration inhibition, although PHA heated at 56°C for 30 min had the same effect in the test as non-heated PHA. This suggests that PHA did not induce migration inhibition by acting directly on the polymorphs, but by activating the pre-incubated lymphocytes to produce MIF.

In the present experiments, the immunologically active

TABLE 3

Migration Inhibition of Non-separated Leukocytes Mixed with Varying Numbers of PHA-Preincubated Mononuclear Leukocytes.

Subject no.*	Migration indices			
	Percentages of PHA-preincubated mononuclear leukocytes in the mixed cell population			
	32 %	16 %	8 %	4 %
10	0.52	0.58	0.64	0.88
11	0.53	0.58	0.56	0.72
19	0.41	0.52	0.52	0.70
20	0.56	0.51	0.50	0.69
28	0.43	0.45	0.47	0.72
35	0.35	0.35	0.42	0.59

* The subjects are numbered as in Table 1.

TABLE 4

Effect of Heating the PHA-Preincubated Mononuclear Leukocytes at 56° C for 30 min.

Subject no.*	Migration indices	
	Non-heated mononuclear leukocytes	Heated mononuclear leukocytes
2	0.69	0.95
17	0.52	0.96
18	0.52	1.08
19	0.52	1.02
26	0.47	1.00
27	0.45	1.00
31	0.43	0.98
33	0.38	0.99
35	0.35	1.03
36	0.33	1.04

* The subjects are numbered as in Table 1.

lymphocytes as well as the migrating polymorphs were obtained from the same subject. Therefore, if PHA did not induce migration inhibition of the leukocytes from a patient, this could be caused not only by non-responsive lymphocytes, but also by a reduced MIF sensitivity of the polymorphs. Such indicator cell problems can be avoided by testing the effect of PHA-preincubated mononuclear leukocytes on non-separated leukocytes or purified polymorphs from normal blood donors.

In consequence, the test presented in this paper has two advantages compared with the PHA leukocyte migration test described by Morison (8). First, a direct (and immunologically irrelevant) effect of PHA on the migration of polymorphs is avoided, since PHA is added only to the mononuclear leukocytes. Second, a deficient MIF production can be distinguished from an impaired MIF sensitivity of the polymorphs by using non-separated leukocytes from normal blood donors as migrating indicator cells.

The present PHA migration test may be useful in the evaluation of the T lymphocyte function in patients with abnormalities of cell-mediated immunity caused by disease or by treatment with glucocorticoids or cytostatics. However, the standard procedure may not be sensitive enough to demonstrate a slight depression of the T cell function. But the present experiments suggest that a better quantitation is obtained if various doses of PHA are used and if varying numbers of PHA-preincubated mononuclear leukocytes are added to the migrating indicator cells.

SUMMARY

An *in vitro* test able to demonstrate phytohemagglutinin (PHA)-induced migration inhibition of human peripheral blood leukocytes was investigated. The migration inhibition was demonstrated by the agarose migration technique. Purified mononuclear leukocytes which had been incubated for 30 min with PHA, then washed and added to non-separated leuko-

cytes induced migration inhibition of the mixed cell population. Since no PHA was added to the non-separated leukocytes, a direct PHA effect on polymorph migration was avoided. If the PHA-preincubated mononuclear leukocytes were heated at 56°C for 30 min, their ability to cause migration inhibition was abolished. This suggests that the inhibition was caused by migration inhibitory factor (MIF) produced by the PHA-stimulated lymphocytes.

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