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INHIBITION OF LEUCOCYTE MIGRATION IN MAN BY THE AGAROSE PLATE TECHNIQUE USING PROTEIN PURIFIED DERIVATIVES (PPD) FROM THREE DIFFERENT SOURCES

By

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The leucocyte migration test as a measure of delayed hypersensitivity in man has been widely used in many laboratories in recent years. However, the results reported are not quite concordant.

Several workers have shown a good correlation between inhibition of leucocyte migration and delayed skin reactions (1, 2, 4, 5, 16, 18, 21). On the other hand, other workers were not able to demonstrate a close correlation between the degree of skin reactivity and the extent of leucocyte inhibition (19), or even between the leucocyte migration test and positive or negative delayed skin reactions (14, 15). Lack of reproducibility and of reliability in interpretation of the results have also been observed (3, 13).

It has been suggested that the capillary tube technique for demonstrating antigen-induced leucocyte migration inhibition gives reproducible results chiefly when particulate antigens are used. Soluble antigens are less effective and high concentrations of antigens are required to obtain good results (14, 15, 22).

In 1971 Clausen proposed an agarose plate technique as an alternative method to the capillary tube one. The agarose

plate technique, compared to the capillary tube method, seems to be more sensitive—chiefly when soluble antigens are used—, more economical in cells and antigens, and easier and quicker to perform.

This study was carried out in order to confirm the reproducibility and sensitivity of the method with soluble antigens (Protein Purified Derivative—PPD), and to evaluate the possibility of using it in clinical practice.

MATERIAL AND METHODS

Our study was carried out on a group of 20 Mantoux-positive and a group of 17 Mantoux-negative persons.

Intracutaneous tuberculin reactions were regarded as positive when an induration of 5 mm or more was observed 72 hours after the intradermal injection of 5 I.U. or 10 I.U. of PPD. Mantoux-negative people were considered those with negative reactions to both intradermal tests. All intradermal tests were carried out after collection of blood samples for migration inhibition tests.

The agarose medium was prepared every day according to the method proposed in 1971 by Clausen (6). Briefly, agarose, TC medium 109 (Difco), horse serum and distilled water were added in proportions to yield a final solution containing 1.00 per cent agarose, 10 per cent horse serum and single strength TC medium 109. The pH was adjusted to between 7.2 and 7.4 by addition of a solution of sodium bicarbonate (10 per cent Difco).

10 ml agarose medium were poured into glass Petri dishes (5 cm diameter) and, after cooling, 10 holes of 2 mm diameter were cut in each plate using a stainless steel punch connected to a suction pump.

Leucocytes were obtained by spontaneous sedimentation of heparinized venous blood. After sedimentation for one hour at 37° C, the leucocyte rich plasma was removed, centrifuged at $220 \times g$ for 5 min and the cell pellet washed twice in TC 109, a third wash being done in TC 109 containing 10 per cent horse serum. After the last wash, the leucocytes were counted in a Burkner chamber. TC medium containing 10 per cent horse serum was then added in order to obtain a final concentration of 2.2×10^6 leucocytes per ml. The average ratio between polymorphonuclear and mononuclear cells was 64 to 36. There were about 2 red blood cells for each leucocyte (the average differential count being 68 per cent r.b.c. and 32 per cent leucocytes). The final cell suspension contained about 96–98 per cent living cells as evaluated by the dye exclusion method. The cells were then incubated at 37° C for ½ hour in the presence of antigen, or for control without antigen, before being placed in the holes in the agarose plates.

It is important to cut holes just before placing the cells, in order to avoid liquid filling the holes and thus preventing equal distribution of the cells.

By means of a calibrated microcapillary, 7 μ l leucocyte suspension containing about 1.5×10^6 cells were placed in each hole in the agarose plates. All tests were carried out at least in triplicate. Two holes in each plate served as control, whereas antigen-preincubated cell suspensions were placed in the remaining pairs. The agarose plates were then incubated for 20 hours at 37° C and the migration haloes recorded by photography and measured with a planimeter.

Migration Indexes (M.I.) were expressed as a ratio between the average area of migration in the presence of antigen and the average area in control cultures according to the following formula:

$$\text{M.I.} = \frac{\text{mean area of migration in the presence of antigen}}{\text{mean area of migration in the absence of antigen}} \times 100$$

In some instances the cells were carefully removed from the plate after migration, stained with May Grünwald Giemsa and observed under high magnification.

The following three different preparations of PPD were used as antigens in various amounts:

PPD Sclavo (Siena, Italy) concentration in leucocyte suspensions was 100, 200, 300 or 400 μ g/ml;

PPD Weybridge (England) was employed at 100 or 200 μ g/ml;

PPD from the State Serum Institute—S.S.I. (Copenhagen, Denmark) at 100 μ g/ml.

RESULTS

As shown in Table 1, the migration of leucocytes from Mantoux-negative persons was not inhibited in the presence of PPD (100, 200 and 300 μ g/ml of PPD Sclavo; 100 and 200 μ g/ml PPD Weybridge; and 100 μ g/ml PPD S.S.I.). With the highest doses of antigen (400 μ g/ml), on the other hand, an inhibitory effect was observed (mean M.I. = 84.89 per cent). The difference between M.I. using 100 μ g/ml or 400 μ g/ml of PPD (Sclavo) was statistically significant ($t = 2.3647$; $P < 0.05$). This effect on leucocytes from Mantoux-negative subjects seems to be due to a nonspecific effect of high concentration of antigen. However, in some instances M.I. higher than 80 per cent were observed (see Table 1).

M.I. of leucocytes from Mantoux-positive subjects in the presence of various doses of PPD from different sources are reported in Table 2 and summarized in Figs. 1, 2 and 3.

TABLE 1
Migration Indexes (M.I.) in 17 Mantoux-negative persons.

No.	Doses of P.P.D. ($\mu\text{g/ml}$)							P.P.D. S.S.I.
	P.P.D. Slavo			P.P.D. Weybridge				
	100	200	300	400	100	200	100	
1	87.80							
2	103.51	105.87						
3	96.67	78.06		63.22				
4	88.73	92.39		72.08				
5	84.71	86.21	85.86	79.32				
6	100.82	101.46	98.12	81.26				
7	100.28	103.09	95.06	87.04				
8	79.74	90.51	90.94	68.25	90.86	87.91		91.13
9	122.55	117.17	102.24	110.38	112.57	108.19		108.50
10	106.30	101.64	83.89	82.52	93.31	90.27		92.41
11		104.89	109.29	110.98	106.49	109.08		105.78
12	118.64	113.81	107.32	93.92	—	112.41		—
13	106.00				104.02	100.43		96.31
14					94.12	89.36		98.11
15					106.01	99.09		108.24
16					88.41	87.24		90.43
17					98.23	102.35		101.16
Mean value	99.64	99.55	96.59	84.89	99.33	98.63		99.11
S.D.	± 13.10	± 14.08	± 9.40	± 16.25	± 8.28	± 9.47		± 7.18

Leucocytes are pre-incubated at 37°C for 30 min in the presence of various doses of P.P.D.

No.	Doses of P.P.D. ($\mu\text{g/ml}$)						
	P.P.D. Slavo			P.P.D. Weybridge			S.S.I.
	100	200	300	400	100	200	
1	75.29						
2	86.72	68.07		59.17			
3	73.14	75.86		61.89			
4	98.39	96.76	80.00	82.18			
5	84.64	88.70	80.75	64.77			
6	102.73	93.05	43.12	43.12			
7	94.37	91.21	87.13	72.14			
8	94.05	93.31	76.09	69.68			
9		102.51	99.49	100.97	100.28		
10			74.98	65.61	73.51	78.79	
11			81.06	66.37	72.26	76.92	
12			78.72	85.60	81.75	72.32	
13			90.74	97.33	98.61	102.81	97.89
14			92.05	79.83	73.35	72.63	85.13
15			73.84	81.23	69.83	64.51	79.17
16				82.04	97.98	80.82	78.27
17				68.71	74.21	68.41	71.67
18				62.50	84.78	85.04	86.92
19				67.17	45.99		63.62
20							84.23
Mean value	88.16	88.68	79.83	72.79	79.32	78.02	80.86
S.D.	± 10.91	± 11.36	± 13.94	± 14.06	± 15.94	± 11.23	± 10.33

Leucocytes are pre-incubated at 37°C for 30 min in the presence of various doses of P.P.D.

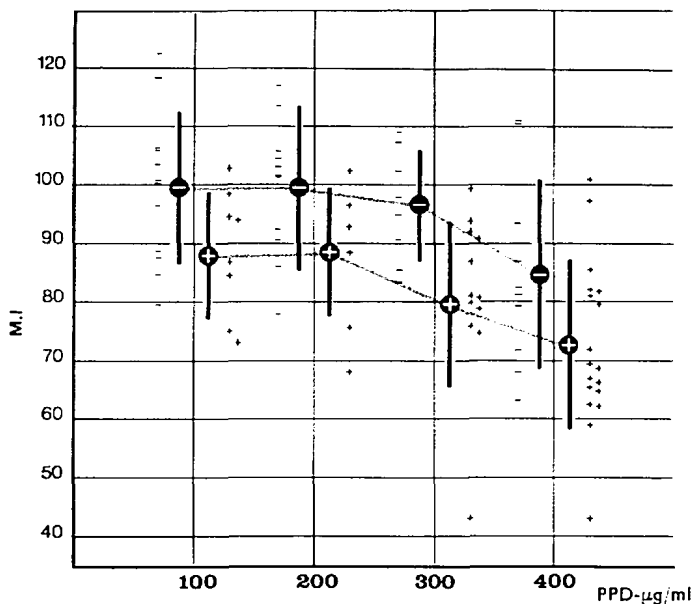


Fig. 1.

Migration Index (M.I.) in Mantoux-positive (+) and Mantoux-negative(—) subjects in the presence of various doses of PPD Sclavo. The circles indicate the mean value of M.I. for each dose of antigen; vertical lines represent the Standard Deviation ($\pm$ s.d.).

The mean value of M.I. for 8 Mantoux-positive subjects in the presence of 100 and 200 μg PPD (Sclavo)/ml was 88.16 (s.d. $\pm$ 10.91) and 88.68 (s.d. $\pm$ 11.36) respectively. The difference between these two M.I. and the corresponding M.I. observed in Mantoux-negative subjects was not statistically significant. When PPD (Sclavo) concentration in leucocyte suspensions was 300 μg /ml, the mean M.I. obtained in 12 Mantoux-positive persons was 79.83 per cent (s.d. $\pm$ 13.94). The difference between the latter and the mean value of M.I. in Mantoux-negative subjects (challenged with 300 μg PPD (Sclavo)/ml) was statistically significant ($t=3.1489$;

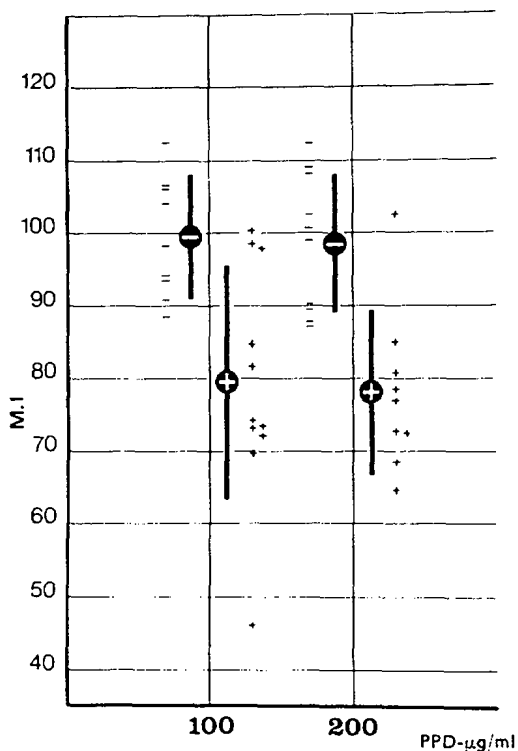


Fig. 2.

M.I. in Mantoux-positive (+) and Mantoux-negative (—) subjects in the presence of various doses of PPD Weybridge. The circles indicate the mean value of M.I. for each dose of antigen; vertical lines represent $\pm$ s.d.

$0.001 < P < 0.01$). The mean value of M.I. in 18 Mantoux-positive subjects tested with 400 µg PPD (Sclavo)/ml was 72.79 per cent (s.d. ± 14.06). The difference between this M.I. and the corresponding M.I. obtained in Mantoux-negative subjects was barely significant ($t = 2.0648$; $0.02 < P < 0.05$).

Eleven and 9 tuberculin-positive subjects were tested using

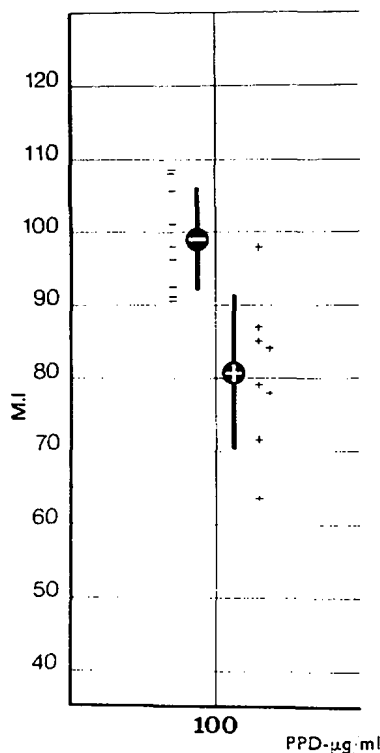


Fig. 3.

M.I. in Mantoux-positive (+) and Mantoux-negative (—) subjects in the presence of PPD from Statens Serum Institute. The circles indicate the mean value of M.I.; vertical lines represent $\pm$ s.d.

100 and 200 μg PPD (Weybridge)/ml respectively. The mean value of M.I. ($79.32 - \text{s.d.} \pm 15.94$ —and $78.02 - \text{s.d.} \pm 11.23$ respectively) was significantly lower than the corresponding M.I. from Mantoux-negative persons ($t = 3.3972$; $0.0001 < P < 0.01$ and $t = 4.0332$; $P < 0.001$).

Eight tests were performed using PPD from S.S.I. (100 $\mu\text{g}/\text{ml}$). The mean value of M.I. was 80.86 (s.d. ± 10.33)

and the comparison with the corresponding M.I. obtained in Mantoux-negative subjects was statistically significant ($t = 4.9768$; $P < 0.001$).

The migration area contained leucocytes exclusively, erythrocytes being observed only at the bottom of the holes cut in the agarose gel. The cells clearly migrated between the agarose and the surface of the Petri plate. The migratory leucocytes were both granulocytes and mononuclear cells; the latter were found to predominate at the periphery of haloes while the former were in prevalence near the holes. When the cells were allowed to migrate for more than 24 hours, the migration area grew larger and thinner, on account of either progressive mononuclear cell migration or the disappearance of most granulocytes.

DISCUSSION

Our results confirm that the agarose plate technique proposed by Clausen (6) for the demonstration of antigen-induced migration inhibition of peripheral blood leucocytes is reproducible, easy to carry out and offers some advantages in comparison with the capillary tube method.

The most important advantage would be the possibility of saving antigen. In fact, despite the high concentration needed in leucocyte suspensions, small amounts of antigen are enough to obtain the required concentrations.

The agarose plate technique seems also to be more sensitive than the capillary tube one (9). This is probably due chiefly to the fact that the leucocytes migrate more quickly in agarose medium during the first 4 hours of culture than they do from capillaries, and are therefore more sensitive to MIF effect (8). In fact it has been suggested by Clausen (8) that the MIF sensitivity of migrating polymorphonuclear cells progressively decreases during the culture period.

However, the specificity of the method was not completely confirmed. Our results correlate well with skin reactivity and are statistically significant when groups of Mantoux-positive

or Mantoux-negative subjects are considered, but M.I. of individual persons are not always correlated with the skin test results, even when high concentrations of PPD are used in culture (Tables 1 and 2 and Figs. 1, 2 and 3).

Furthermore, only high doses of PPD, close to "toxic" doses, give low M.I. closely correlated with positive Mantoux tests. This makes the method somewhat difficult to employ with soluble antigens. It may also be observed that different antigen preparations give different results. In this connection, the same source of antigen should be used in order to compare data obtained by different workers. In our previous experiments, we were unable to confirm Clausen's results when using the same amount of PPD (Sclavo) in cell suspensions. However, during the preparation of this manuscript Clausen published a study on the effect of varying doses of PPD on the migration of human peripheral blood leucocytes (11). Our results are similar to those obtained by Clausen in Mantoux-positive persons with high doses of PPD (100, 200, 400 μg PPD from S.S.I./ml), but mean M.I. as well as single M.I. are higher than those observed by Clausen. It should be noted that the PPD we used at high concentration (PPD from Sclavo and Weybridge) was different from that employed by Clausen.

In Mantoux-negative subjects (only one case is reported by Clausen), mean M.I. with 200 and 300 μg /ml of PPD Sclavo and with 200 μg /ml of PPD Weybridge are similar to those obtained with 100 μg /ml of PPD Sclavo and Weybridge. Only with 400 μg /ml of PPD Sclavo was there a significant decrease in M.I. However, considering the single M.I., 4 were lower than 80 per cent while 6 were higher than 80 per cent, ranging from 81.26 per cent to 110.98 per cent. A slight non-specific ("toxic"?) effect may therefore be present at this dose of antigen.

According to our observations, the inhibited cells seem to be mononuclear leucocytes. These cells are found at the periphery of haloes and seem to be affected by MIF, whereas the polymorphonuclear leucocytes always remain near the

holes in inhibited and uninhibited areas. Similar observations were made by Rosenberg & David (19) and by Falk (12) using the capillary tube technique. It is worth noting that in the capillary tube migration test the mononuclear cells are mainly found at the center of haloes (20).

It has been demonstrated that homologous IgG (γ_2 subclass) may cause leucocyte inhibition in the presence of specific antigen (17). This observation leads us to the question of the immunological significance of the method. In this regard, Clausen reported that significant leucocyte migration inhibition could be observed either in the presence of cell-free supernatants from mixed human lymphocyte cultures (7) or in the presence of cell-free supernatants from tuberculin-stimulated cultures of human mononuclear leucocytes (10). Similar results are confirmed by Weisbart et al. (22), who also provide some evidence for antigen dependency of MIF in leucocyte migration inhibition in the presence of PPD. These data seem to exclude the effect of cell-bound antibodies on migration of peripheral blood leucocytes. Furthermore, in our studies as well as in those of Clausen (6), Astor et al. (1) and Weisbart et al. (22), circulating or cell-bound antibodies are not likely to be involved, since the cells are washed several times and resuspended in inactivated heterologous serum, before being placed in migration plates.

Although several years have passed since the first description of the method of leucocyte migration inhibition in the presence of soluble antigens, further studies seem to be needed in order to establish definitively its usefulness and immunological significance.

SUMMARY

In vitro response to Protein Purified Derivative (PPD) was evaluated by the leucocyte migration inhibition test with the agarose plate technique. Three different sources of PPD were used, and some differences were observed with different preparations. A dose-response curve was studied in Mantoux-posi-

tive and Mantoux-negative subjects with one preparation of PPD. A good correlation between *in vitro* tests and skin tests was observed, although single *in vitro* and *in vivo* reactions were not always concordant.

REFERENCES

1. Astor, S. H., Spitler, L. E., Frich, O. L. & Fudenberg, H. H. (1973): Human leucocyte migration inhibition in agarose using four antigens: Correlation with skin reactivity. *J. Immunol.* **110**, 1174-1179.
2. Bendixen, G. & Søborg, M. (1970): Comments on the leucocyte migration technique as an *in vitro* method for demonstration of cellular hypersensitivity in man. *J. Immunol.* **104**, 1551-1552.
3. Brogan, T. D. (1972): Leucocyte migration test. *Lancet* **i**, 537-538.
4. Brostoff, J. & Roitt, I. M. (1969): Cell-mediated (delayed) hypersensitivity in patients with summer hay-fever. *Lancet* **ii**, 1269-1272.
5. Clausen, J. E. & Søborg, M. (1969): *In vitro* detection of tuberculin hypersensitivity in man. Specific migration inhibition of white blood cells from tuberculin-positive persons. *Acta Med. Scandinav.* **186**, 227-230.
6. Clausen, J. E. (1971): Tuberculin-induced migration inhibition of human peripheral leucocytes in agarose medium. *Acta Allergol.* **26**, 56-80.
7. Clausen, J. E. (1972): Migration inhibitory effect of cell-free supernatants from mixed human lymphocyte cultures. *J. Immunol.* **108**, 453-459.
8. Clausen, J. E. (1973): Tuberculin-induced migration inhibition of human peripheral blood leucocytes after 2, 4 and 20 hours in agarose culture. *Acta Allergol.* **28**, 28-41.
9. Clausen, J. E. (1973): Comparison between capillary tube and agarose migration technique in the study of human peripheral blood leucocytes. *Acta Allergol.* **28**, 145-158.
10. Clausen, J. E. (1973): Migration inhibitory effect of cell-free supernatants from tuberculin stimulated cultures of human mononuclear leucocytes demonstrated by two step MIF agarose assay. *J. Immunol.* **110**, 546-551.
11. Clausen, J. E. (1973): Dose effect relationship between tuberculin concentration and tuberculin induced migration inhibition of human peripheral blood leucocytes demonstrated by agarose migration techniques. *Acta Allergol.* **28**, 159-171.
12. Falk, R. E. (1971), In: *In vitro* methods in cell-mediated immunity. I. Methods for study mediators (Ed. B. R. Bloom and P. R. Glade). Academic Press, New York and London. pp. 22-23.
13. Foulis, A. K. (1972): Leucocyte migration test. *Lancet* **i**, 842-843.
14. Kaltreider, H. B., Soghor, D., Taylor, J. B. & Decker, J. L. (1969): Capillary tube migration for detection of human delayed hypersensitivity: diffi-

- culties encountered with "buffy coat" cells and tuberculin antigen. *J. Immunol.* 103, 179-183.
15. Lockshin, M. D. (1969): Failure to demonstrate leucocyte migration inhibition in human tuberculin hypersensitivity. *Proc. Soc. Exp. Biol. Med.* 132, 928-930.
 16. Muller-Berat, C. N. & Moulias, R. (1970): Le test de migration des leucocytes du sang périphérique (T.M.L.). Un nouveau test d'hypersensibilité retardée in vitro chez l'homme. *Presse Med.* 78, 77-79.
 17. Packalen, T. & Wasserman, J. (1971): Inhibition of migration of normal guinea pig blood leucocytes by homologous immune γ_2 globulin in the presence of specific antigen. *Int. Arch. All. Appl. Immunol.* 41, 790-796.
 18. Rauch, H. C. & King, K. (1973): Human leucocyte migration inhibition as an indicator of cellular hypersensitivity to soluble antigens. *Int. Arch. All. Appl. Immunol.* 44, 862-874.
 19. Rosenberg, S. A. & David, J. R. (1970): Inhibition of leucocyte migration: an evaluation of this in vitro assay of delayed hypersensitivity in man to a soluble antigen. *J. Immunol.* 105, 1447-1452.
 20. Rosenberg, S. A. (1971), In: *In vitro methods in cell-mediated immunity. I. Methods for study mediators* (Ed. B. R. Bloom and P. R. Glade). Academic Press, New York and London. pp. 19-21.
 21. Søborg, M. & Bendixen, G. (1967): Human lymphocyte migration as a parameter of hypersensitivity. *Acta Med. Scandin.* 181, 247-255.
 22. Weisbart, R. H., Cunningham, J. E., Bluestone, R. & Goldberg, L. S. (1973): A modified agarose method for detection of migration inhibitory factor and delineation of its antigen dependency. *Int. Arch. All. Appl. Immunol.* 45, 612-619.

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