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TUBERCULIN-INDUCED MIGRATION INHIBITION OF HUMAN PERIPHERAL LEUCOCYTES IN AGAROSE MEDIUM

By

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INTRODUCTION

In 1928, *Rich & Lewis* (6) showed that tuberculin inhibited migration of cells from animals with delayed hypersensitivity to tuberculin. Cells from buffy coat or small splenic fragments were used as migratory cells.

Since then antigen-induced inhibition of cell migration has been a method frequently employed for the in vitro demonstration of cellular hypersensitivity.

Technically, it was a considerable advance when *George & Vaughan* (5) introduced a capillary tube technique to demonstrate antigen-induced migration inhibition. In the capillary tube technique the cells are transferred to a capillary tube which is placed in a culture chamber. The cells migrate from the tube opening as a fine sheet along the bottom of the chamber.

In 1967, *Søborg & Bendixen* (7) showed that the capillary tube technique as employed earlier in animal investigations, especially by *David et al.* (4), could be used to demonstrate cellular hypersensitivity in man, using leucocytes as migratory cells. *Clausen & Søborg* (3) have demonstrated by this technique, tuberculin-induced inhibition of migration of leucocytes from tuberculin-positive persons.

Carpenter et al. (2) have shown that in animal investigations, an agar medium containing agar and guinea-pig serum in a tissue culture medium supported migration of cells from fragments of spleen, lung and lymph node, and from suspensions of splenic or peritoneal cells. In experiments using cell suspensions the cells were placed in holes cut in the agar gel. The migration was predominantly between the agar and the petri dish containing the agar. Migration of cells from sensitized animals was specifically inhibited, when antigen was added to the agar medium.

The purpose of the present study was to investigate whether an agar medium could be used for examinations of antigen-induced migration inhibition of human leucocytes.

MATERIALS AND METHODS

The material comprised 56 tuberculin-positive and 15 tuberculin-negative persons.

Tuberculin-positive persons are defined as those where the intradermal tuberculin test showed an induration of 5 mm or more 72 hours after the injection of 0.1 ml tuberculin solution (State Serum Institute, Copenhagen, Denmark) containing 0.02 μ g purified protein derivative (PPD) (Mantoux I test) or 0.2 μ g PPD (Mantoux II test). None of the persons showed signs of active tuberculous infection. Some were vaccinated with BCG, others were spontaneous tuberculin-positive.

Tuberculin-negative persons are defined as those where both intradermal tests were negative.

Patients with diseases which are supposed to have an influence on the cellular hypersensitivity, and patients under treatment with glucocorticoids or cytostatics were not included in the study.

Investigations of migration were always carried out before intradermal tuberculin tests.

Preparation of Agarose Medium

Fresh agarose medium was prepared every day. A 2 per cent solution of agarose (Litex, Glostrup, Denmark) after cooling to 47° C, was mixed with 47° C fetal calf serum (Flow Laboratories, Irvine, Scotland) or horse serum (State Serum Institute, Copenhagen, Denmark), sterilized water and a ten-fold concentration of tissue culture medium 199 (TC-medium 199, 10 $\times$ from Difco Laboratories, Detroit, Michigan, U.S.A.) in such proportions that the final solution contained either 0.75 per cent agarose and 10 per cent fetal calf serum

in single strength TC medium 199, or 1.00 per cent agarose and 10 per cent horse serum in single strength TC medium 199. Penicillin and streptomycin (TC penicillin-streptomycin, desiccated from Difco) was added, so that the concentration of penicillin was 66 units per ml and the concentration of streptomycin 66 μg per ml. Sodium bicarbonate solution (TC sodium bicarbonate solution 10 per cent from Difco) was added, so that pH in the agarose medium, after incubating in 2 per cent CO_2 , 98 per cent atmospheric air saturated with water vapor was between 7.2 and 7.4.

In some cases PPD (a solution containing 1 mg PPD per ml phosphate buffer without preservative, from The State Serum Institute, Copenhagen, Denmark) or, as control, phosphate buffer was added to the agarose medium, so that the final PPD concentration was 50 or 100 μg per ml.

5 ml agarose-serum-TC medium 199 was poured into disposable plastic petri dishes (Millipore, Bedford, Mass., U.S.A.). After a gel had formed, 8 holes 2.3 mm diameter were cut in each agarose plate, using a stainless steel punch (Fig. 1).

Preparation of Leucocyte Suspensions

Venous blood was collected in 10 ml polystyrene tubes (Nuncion tubes from Nunc, Roskilde, Denmark), each containing 250 i.u. heparin (heparin with 5000 i.u. per ml from Leo, Copenhagen, Denmark).

In most cases the blood sedimented spontaneously but in few cases where the sedimentation rate was low, the blood was mixed with 5 per cent dextran 250 (dextran 250 with mol. wt. 250,000 from Pharmacia, Uppsala, Sweden), in saline solution in the proportion 4:1. After sedimentation for 1 hour at 37° C, the supernatant leucocyte rich plasma was removed, centrifugated at 220 g for 5 minutes and the cell pellet washed three times in Hanks balanced salt solution. The washed leucocytes were then resuspended in TC medium 199 (Difco) with 10 per cent serum added to give a final concentration of 2.2×10^6 leucocytes per ml. The leucocyte yield was about 45×10^6 leucocytes per 10 ml of blood. The average differential count was 35 per cent mononuclear and 65 per cent polymorphonuclear leucocytes.

Leucocyte Migration using Agarose Plate Technique

In most of the tests, PPD was added to half of the leucocyte suspension and, as control, the same amount of phosphate buffer to the other half. The PPD concentration in the leucocyte suspension was 12.5, 25, 50 or 100 $\mu\text{g}/\text{ml}$. Thereafter, the leucocyte suspensions were incubated at 37° C for $\frac{1}{2}$ hour before being placed in holes in agarose plates which contained neither PPD nor phosphate buffer. In every hole in the agarose plates 7 μl leucocyte suspension containing 1.5×10^6 leucocytes was placed. In 4 of the holes in an agarose plate, leucocytes preincubated with PPD in phosphate buffer, were placed, and in the remaining 4 holes, as control, leucocytes preincubated with phosphate buffer.

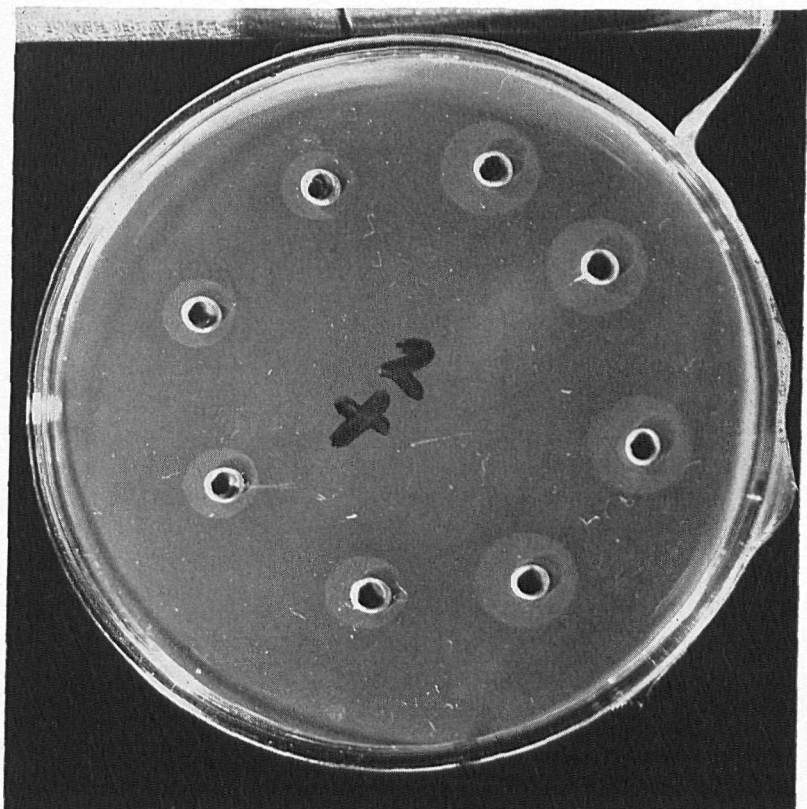


Fig. 1.

Petri dish with agarose medium. Areas of migration are seen around the holes which contain leucocyte suspension. Tuberculin-induced migration inhibition of the 4 cultures on the left is clearly visible.

In some tests, the leucocyte suspension without the addition of PPD or phosphate buffer was placed immediately in holes in agarose plates containing 50 or 100 μg PPD per ml or, as control, a corresponding amount of phosphate buffer.

The agarose plates were then incubated for 24 hours at 37° C, in 2 per cent CO_2 , 98 per cent atmospheric air saturated with water vapor. After 24 hours, the migration areas were studied under a projection microscope and measured by planimetry. Generally, 8 control cultures and 8 cultures with tuberculin were prepared. The degree of migration inhibition was expressed as a migra-

tion index (MI), as a ratio between the average area of tuberculin containing cultures and control cultures.

Leucocyte Migration using Capillary Tube Technique

This technique has been described in detail earlier by *Søborg & Bendixen* (1, 7). As the culture medium, TC medium 199 (Difco) with 10 per cent horse serum added, was used. The leucocytes were washed 3 times, drawn into capillary tubes and placed in 1 ml culture chambers. 100 μ l PPD solution containing 100 μ g PPD was added to half of the chambers and 100 μ l phosphate buffer to the other half.

In some cases, TC medium 199 containing 10 per cent fetal calf serum was used.

In a few investigations, leucocytes preincubated for $\frac{1}{2}$ hour with PPD 50 μ g/ml or a corresponding amount of phosphate buffer were drawn into capillary tubes and placed in culture chambers with TC medium 199 containing 10 per cent horse serum but no PPD or phosphate buffer.

The migration areas were measured after 24 hours and the MI was calculated as described above.

RESULTS

Fig. 1 shows an agarose plate with migration areas from leucocyte suspensions preincubated with PPD or phosphate buffer. The 4 large migration areas are from control cultures and the 4 small are from cultures in which the leucocytes have been preincubated for $\frac{1}{2}$ hour with PPD 50 μ g/ml.

The migratory cells were only found, apparently, between the agarose gel and the petri dish.

The cells in the migration area beneath the agarose gel contained only leucocytes and no erythrocytes. The leucocytes in the migration area consisted of both granulocytes and mononuclear cells and both cell types were represented in the whole migration area.

In some tests, 7 μ l pure erythrocyte suspension containing 1.5×10^6 erythrocytes was placed in holes in the agarose plate. In none of these tests were erythrocytes observed beneath the agarose gel after 24 hours. This indicates that the spreading of the cells beneath the agarose gel is caused only by the cell migration and not passive sliding out of the cells.

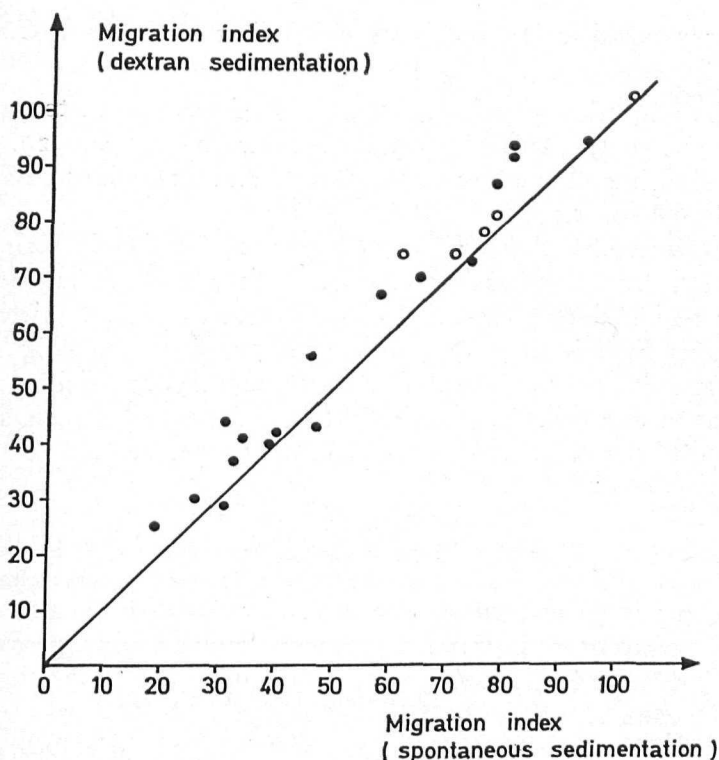


Fig. 2.

Comparison between migration indices using dextran sedimentation and spontaneous sedimentation of blood. The leucocytes were preincubated with PPD 50 $\mu\text{g}/\text{ml}$ and tested on agarose plates containing horse serum (17 cases, ●) or fetal calf serum (5 cases, ○). The line $y = x$ is drawn.

In 22 cases MI for leucocytes preincubated with PPD 50 $\mu\text{g}/\text{ml}$ was assessed both after spontaneous sedimentation and after dextran sedimentation of the blood.

Fig. 2 shows a comparison between MI using spontaneous sedimentation and MI using dextran sedimentation.

MI using dextran sedimentation gave an average of 0.05 larger than MI using spontaneous sedimentation. The difference was small, but statistically significant, assessed by means of paired comparisons ($p < 0.001$).

Leucocyte Migration in Agarose Medium with Fetal Calf Serum.

The migration area varied somewhat from person to person. The control cultures had areas between 9.91 and 29.37 mm² showing an average of 18.38 mm² and a standard deviation (SD) of 5.22.

To determine the MI for each person, 8 control cultures and 8 cultures containing tuberculin were used. The coefficient of variation for the 8 control cultures in a MI determination was between 2.7 and 15.8 per cent, showing an average of 7.1 per cent. The corresponding coefficient of variation for cultures containing tuberculin was between 2.0 and 16.6 per cent, with an average of 7.1 per cent.

TABLE 1

Migration indices of leucocytes from 11 persons. Leucocytes were preincubated varying time with PPD 50 µg/ml before application in agarose medium without PPD (0.75 per cent agarose, 10 per cent fetal calf serum in TC 199).

Leucocytes applied just after addition of PPD	Leucocytes preincubated with PPD		
	½ h	1½ h	2½ h
0.96	1.00	0.95	—
1.10	0.99	1.07	—
—	0.74	0.76	—
0.67	0.72	0.59	—
0.80	0.72	0.74	0.88
0.81	0.70	0.73	—
0.83	0.64	0.81	—
—	0.59	0.75	—
0.59	0.57	0.50	—
0.60	0.55	0.69	0.89
0.54	0.51	0.48	0.68

Investigation of migration with leucocytes preincubated with PPD. Table 1 shows the result of an investigation where leucocytes from 11 persons were incubated for varying time

with PPD 50 $\mu\text{g}/\text{ml}$ before placing on agarose plates. The lowest MI was found in most cases after preincubating for $\frac{1}{2}$ hour and this time was chosen, therefore, as the routine preincubation time for later tests. The difference between MI after 0, $\frac{1}{2}$ and $1\frac{1}{2}$ hours preincubating with PPD was, however, not statistically significant with the method of paired comparisons ($0.05 < p < 0.1$ for the difference between MI after preincubating for 0 and $\frac{1}{2}$ hour; $p > 0.1$ for the difference between MI after preincubating for $\frac{1}{2}$ and $1\frac{1}{2}$ hours). When the concentrated leucocyte suspension (2.2×10^8 leucocytes per ml) was left for more than 3 hours before placing on agarose plates, the migration areas were very small both for control cultures and for cultures containing tuberculin, and the tuberculin-induced migration inhibition could no longer be seen. After preincubating for 6 hours, no migration was seen in either the control cultures or the cultures containing tuberculin.

In 22 cases, MI for leucocytes preincubated with 50 μg PPD/ml was determined twice at intervals of 2 to 11 days. The difference in MI between the two determinations varied between 0 and 0.22, with an average of 0.08. The difference was independent of the mean value of the two determinations. As the 22 persons supposedly found themselves in a steady state as regards tuberculin hypersensitivity, the accuracy of the method can be assessed from these 22 pairs of MI values. 95 per cent confidence limits for a MI determination evaluated from these tests was found to be the observed value ± 0.13 .

Table 2 shows MI from 13 tuberculin-negative and 40 tuberculin-positive persons. The mean value for MI in cultures preincubated with 50 μg PPD/ml was 0.90 with SD 0.10 for tuberculin-negative persons whereas for tuberculin-positive persons the mean value was 0.66 with SD 0.12. The 2 groups were significantly different ($p < 0.001$).

Among the tuberculin-positive, 23 had positive cutaneous reaction after 0.02 μg PPD while 16 did not show positive reaction until after 0.2 μg PPD. The mean values of MI for

TABLE 2

Migration indices from 13 tuberculin-negative and 40 tuberculin-positive persons. Leucocytes were preincubated with PPD and cultured in agarose medium without PPD (0.75 per cent agarose, 10 per cent fetal calf serum in TC 199).

Tuberculin-negative persons		Tuberculin-positive persons				
Leucocytes preincubated with PPD ($\mu\text{g/ml}$)		Cutaneous reaction in mm after PPD (μg)		Leucocytes preincubated with PPD ($\mu\text{g/ml}$)		
50	25	0.02	0.2	50	25	12.5
1.03	1.03	0	6	0.84	—	—
1.02	—	0	7	0.81	—	—
1.00	1.08	0	7	1.02	—	—
0.99	0.95	0	8	0.71	0.76	—
0.96	—	0	9	0.85	0.94	0.95
0.95	—	0	10	0.68	—	—
0.87	—	0	12	0.67	—	—
0.86	0.90	0	12	0.68	—	—
0.84	—	0	14	—	0.68	0.78
0.81	—	0	14	0.55	0.69	—
0.81	—	0	14	0.65	0.57	0.70
0.80	0.94	0	14	0.66	—	—
0.71	—	0	14	0.60	—	—
		0	16	0.75	—	—
		0	16	0.56	—	—
		0	18	0.72	0.88	—
		0	18	0.74	—	—
		7	—	0.62	—	—
		7	—	0.80	—	—
		10	—	0.66	0.57	—
		12	—	0.53	0.59	0.72
		12	—	0.66	—	—
		14	—	0.79	—	—
		14	—	0.73	0.80	—
		14	—	0.70	0.67	0.87
		15	—	0.67	—	—
		15	—	0.64	—	—
		16	—	0.61	—	—
		16	—	0.68	—	—
		17	—	0.55	0.61	—
		17	—	0.72	—	—

TABLE 2. (Cont.)

Tuberculin-negative persons Leucocytes preincubated with PPD ($\mu\text{g/ml}$)		Tuberculin-positive persons				
		Cutaneous reaction in mm after PPD (μg)		Leucocytes preincubated with PPD ($\mu\text{g/ml}$)		
50	25	0.02	0.2	50	25	12.5
		18	—	0.54	0.54	0.64
		18	—	0.58	—	—
		18	—	0.69	—	—
		20	—	0.53	—	—
		21	—	0.70	—	—
		24	—	0.41	—	—
		25	—	0.51	0.69	—
		26	—	0.45	—	—
		30	—	0.46	—	—
Mean						
0.90	0.98			0.66	0.69	0.78
SD						
0.10	0.07			0.12	0.12	0.12

these 2 tuberculin-positive groups were 0.62 with SD 0.11 and 0.72 with SD 0.12 respectively. The 2 groups were significantly different ($p < 0.02$) and both significantly different from the tuberculin-negative group ($p < 0.001$).

Fig. 3 shows migration indices from Table 2 for leucocytes preincubated with PPD 50 $\mu\text{g/ml}$ arranged in 3 groups: Mantoux negative, Mantoux I positive, and Mantoux II positive.

Table 2 shows that leucocytes from tuberculin-positive also showed frequently migration inhibition with 25 and 12.5 μg PPD/ml.

12 tuberculin-positive persons were examined using both 50 and 25 μg PPD/ml. The mean value for MI was 0.64 with SD 0.11 and 0.69 with SD 0.13, respectively. Paired comparisons of MI determined with 50 and 25 μg PPD/ml, showed no significant difference ($0.05 < p < 0.1$).

MIGRATION INDEX

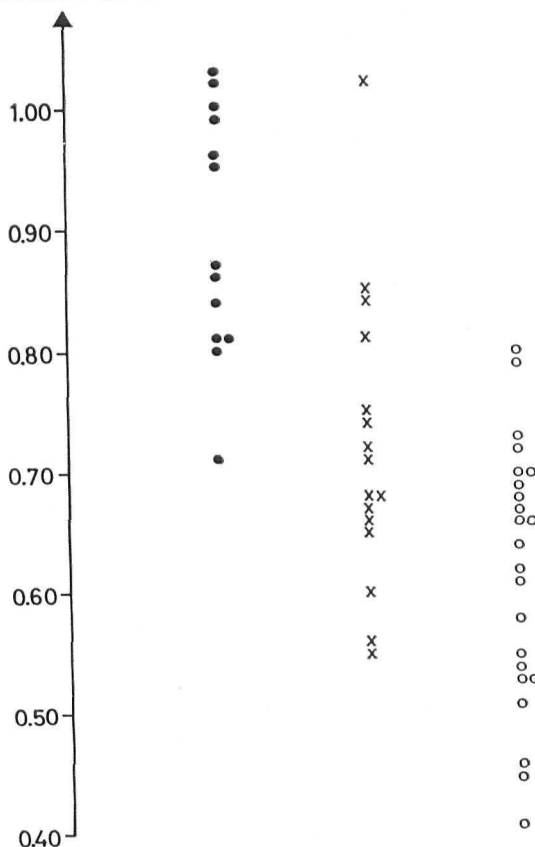


Fig. 3.

Migration indices from tuberculin-negative persons (●), persons with positive Mantoux I (○) and persons with positive Mantoux II (×). The leucocytes were preincubated with PPD 50 $\mu\text{g}/\text{ml}$ and examined in agarose medium containing fetal calf serum.

6 tuberculin-positive persons were examined using both 25 and 12.5 μg PPD/ml. The mean values for MI were 0.66 with SD 0.16 and 0.78 with SD 0.13, respectively. Paired comparisons of MI determined with 25 and 12.5 μg PPD/ml showed a significant difference ($p < 0.01$).

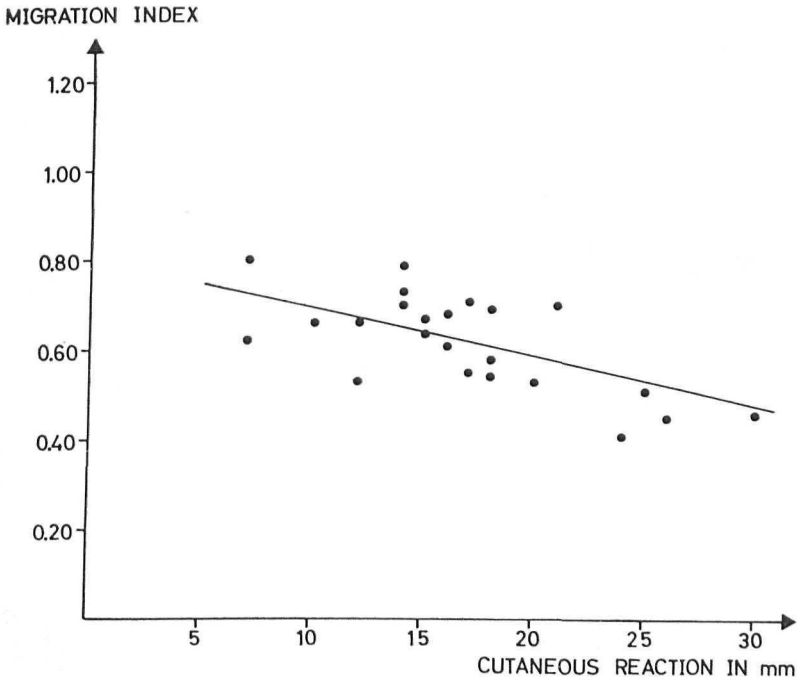


Fig. 4.

23 persons with positive Mantoux I. Correlation of migration index for leucocytes preincubated with PPD 50 $\mu\text{g}/\text{ml}$ and the intracutaneous tuberculin reaction. Leucocyte cultures in agarose medium containing fetal calf serum.

Fig. 4 shows the relationship between the size of the cutaneous reaction and MI determined with leucocytes preincubated with PPD 50 $\mu\text{g}/\text{ml}$ for 23 persons who had positive cutaneous reaction after 0.02 μg PPD. There was good correlation between the 2 parameters ($r = 0.67$, $p < 0.001$).

Fig. 5 shows the relationship between the size of the cutaneous reaction and MI for 16 persons who did not show positive cutaneous reaction until after 0.2 μg PPD. There was correlation between the 2 parameters ($r = 0.59$, $p < 0.05$).

Investigation with non-preincubated leucocytes. Table 3 shows

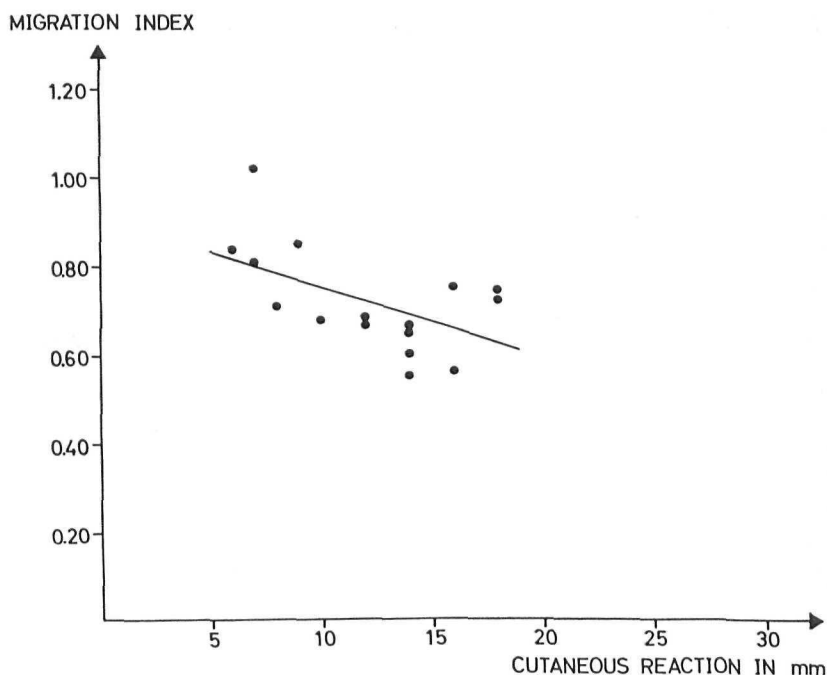


Fig. 5.

16 persons with positive Mantoux II. Correlation of migration index for leucocytes preincubated with PPD 50 $\mu\text{g}/\text{ml}$ and the intracutaneous tuberculin reaction. Leucocyte cultures in agarose medium containing fetal calf serum.

a comparison between MI for 13 persons determined partly with leucocytes without the addition of PPD placed on agarose plates containing PPD 50 or 100 $\mu\text{g}/\text{ml}$, and partly with leucocytes preincubated with PPD 50 $\mu\text{g}/\text{ml}$. For the tuberculin-positive (subjects 2-13) the mean value for MI determined with leucocytes placed on agarose plates containing 50 μg PPD/ml was 0.84 with SD 0.09. The mean value for MI determined with leucocytes preincubated with 50 μg PPD/ml was 0.66 with SD 0.12. The MI in these 2 series differed significantly ($p < 0.001$).

MI for 8 tuberculin-positive persons was determined using agarose plates containing partly 50 and partly 100 μg PPD/

TABLE 3

Comparison of the migration inhibition reaction in agarose medium without PPD using leucocytes preincubated $\frac{1}{2}$ h with PPD, and in agarose medium with PPD using leucocytes without added PPD (0.75 per cent agarose, 10 per cent fetal calf serum in TC 100).

Subject	Agarose medium without PPD Leucocytes preincubated with PPD 50 $\mu\text{g/ml}$	Agarose medium containing PPD ($\mu\text{g/ml}$) Leucocytes without added PPD	
		100	50
1	0.81	0.93	0.94
2	0.84	—	0.77
3	0.81	0.78	0.75
4	0.79	0.88	0.89
5	0.72	0.88	0.89
6	0.69	—	0.78
7	0.68	1.04	1.02
8	0.66	1.03	0.74
9	0.60	0.96	0.90
10	0.58	0.73	0.87
11	0.56	0.78	0.80
12	0.55	—	0.73
13	0.41	—	0.80

ml. The mean values for MI with these 2 PPD concentrations were 0.86 with SD 0.09 and 0.89 with SD 0.12, respectively. The MI for the 2 series were not significantly different ($p > 0.1$).

Leucocyte Migration in Agarose Medium with Horse Serum.

In agarose medium with horse serum, the agarose concentration was 1.00 per cent whereas in medium with fetal calf serum it was 0.75 per cent. The reason for using the higher agarose concentration was that the leucocyte covered migration areas on plates with horse serum were better defined, when the agarose concentration was increased from 0.75 to 1.00 per cent. The migration areas on plates with fetal calf

serum, on the contrary, were well defined with both agarose concentrations.

On agarose plates with horse serum the migration area varied also from person to person. The control cultures had areas between 8.76 and 59.56 mm², showing an average of 31.40 mm² and SD 12.90.

It is seen that the migration areas on agarose plates with horse serum were somewhat larger than the migration areas on plates with fetal calf serum.

The coefficient of variation for the 8 control cultures in the individual migration investigation was between 1.5 and 9.4 per cent, averaging 5.1 per cent. Correspondingly, the coefficient of variation for cultures containing tuberculin was between 1.8 and 13.2 per cent, with an average of 6.2 per cent.

Investigation with leucocytes preincubated with PPD. In 37 cases MI for leucocytes preincubated with 50 µg PPD/ml was determined in duplicate. The difference between the two values from the double determinations varied between 0 and 0.11. The difference was independent of the mean value of the double determination which varied between 0.27 and 0.95. Calculated from these double determinations, 95 per cent confidence limits for a MI determination was found to be the observed value ± 0.06 .

In 12 cases MI for leucocytes preincubated with PPD 50 µg/ml was determined twice with an interval of 1 to 4 days. The difference in MI between 2 determinations varied between 0.01 and 0.13, with an average of 0.05. The difference was independent of the mean value of these two determinations which varied from 0.21 to 0.79. The 12 persons supposedly found themselves to be in a steady state as regards tuberculin hypersensitivity. The accuracy of the method, calculated from these 12 pairs of MI values showed that 95 per cent confidence limits for a MI determination was found to be the observed value ± 0.09 .

Table 4 shows MI for 2 tuberculin-negative and 16 tuberculin-positive persons. The mean value for MI for all the

TABLE 4

Migration indices from 2 tuberculin-negative and 16 tuberculin-positive persons. Leucocytes were cultured in agarose medium (1.00 per cent agarose, 10 per cent horse serum in TC 100).

Cutaneous reaction in mm after PPD (μ g)		Agarose medium without PPD Leucocytes preincubated with PPD (μ g/ml)			Agarose medium containing PPD (μ g/ml) Leucocytes without added PPD	
0.02	0.2	100	50	25	100	50
0	0	—	1.02	—	—	—
0	0	—	0.95	—	—	—
0	6	0.72	0.80	0.85	—	—
0	7	—	0.58	0.73	0.56	0.76
0	12	0.48	0.59	0.72	—	—
0	13	0.62	0.58	0.66	—	—
6	—	—	0.78	0.88	—	—
6	—	0.59	0.65	0.78	—	—
9	—	—	0.81	—	—	1.08
10	—	—	0.71	—	—	1.05
11	—	0.64	0.72	0.88	—	—
14	—	0.49	0.66	0.73	—	—
15	—	0.74	0.82	0.88	—	—
16	—	—	0.50	—	—	—
16	—	—	0.40	0.51	0.57	0.73
21	—	0.34	0.31	0.33	—	—
25	—	—	0.21	0.25	0.62	1.02
26	—	0.25	0.28	0.32	—	—

tuberculin-positive persons tested with leucocytes preincubated with PPD 50 μ g/ml, was 0.59 with SD 0.20. Among the tuberculin-positive persons, 12 showed positive cutaneous reaction after 0.02 μ g PPD, while 4 did not show positive cutaneous reaction until after 0.2 μ g PPD. The mean values for MI for these tuberculin-positive groups were 0.57 with SD 0.22 and 0.64 with SD 0.10, respectively. The 2 groups were not significantly different ($p > 0.1$).

9 tuberculin-positive persons were examined with both 100

and 50 μg PPD/ml. The mean values for MI were 0.54 with SD 0.17 and 0.60 with SD 0.19, respectively. Paired comparison of MI tested with 100 and 50 μg PPD/ml showed a significant difference ($p < 0.05$).

13 tuberculin-positive persons were examined with both 50 and 25 μg PPD/ml. The mean values for MI were 0.57 with SD 0.21 and 0.66 with SD 0.23, respectively. Paired comparisons of MI tested with 50 and 25 μg PPD/ml showed a significant difference ($p < 0.001$).

PPD concentrations 100, 50 and 25 μg PPD/ml gave almost the same migration inhibition among the 3 most intense tuberculin-positive persons (the 3 persons who showed a Mantoux I reaction greater than 20 mm).

A specially intense tuberculin-positive person with a Mantoux I reaction of 26 mm was examined with PPD concentrations 100, 50, 25, 5 and 1 μg PPD/ml. The corresponding MI were 0.25, 0.28, 0.32, 0.56 and 0.80.

The tuberculin-induced migration inhibition of leucocytes preincubated with 50 μg PPD/ml was found to be lower on agarose plates with horse serum than on plates with fetal calf serum. The difference was however not statistically significant ($p > 0.1$).

Fig. 6 shows the relationship between the size of the cutaneous reaction and MI for leucocytes preincubated with PPD 50 $\mu\text{g}/\text{ml}$ for 12 persons who showed positive cutaneous reaction after 0.02 μg PPD. There was good correlation between the 2 parameters ($r = 0.85$, $p < 0.001$).

Investigation with non-preincubated leucocytes. In Table 4 the result of 5 investigations is seen, where MI was determined, partly with leucocytes without the addition of PPD, placed in agarose medium with 50 μg PPD/ml and in 3 cases also in medium with 100 μg PPD/ml, and partly with leucocytes preincubated with PPD 50 $\mu\text{g}/\text{ml}$ placed in medium without PPD. With 50 μg PPD/ml in the agarose medium, no inhibition was found in 3 out of 5 cases. In 2 cases, slight inhibition was found, but clearly less than the inhibition of the same leucocytes preincubated with 50 μg PPD/ml.

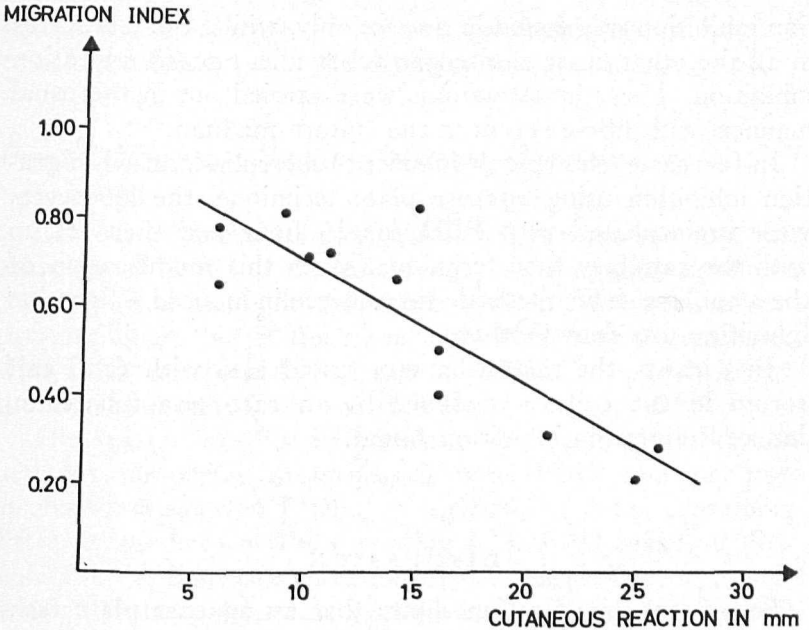


Fig. 6.

12 persons with positive Mantoux I. Correlation of migration index for leucocytes preincubated with PPD 50 $\mu\text{g}/\text{ml}$ and the intracutaneous tuberculin reaction. Leucocyte cultures in agarose medium containing horse serum.

All 3 investigations with 100 μg PPD/ml in the agarose medium clearly showed inhibition, more pronounced than in the corresponding investigations with 50 μg PPD/ml in the medium. In 2 cases the inhibition with 100 μg PPD/ml was clearly less than MI tested with leucocytes preincubated with 50 μg PPD/ml. In the third case, MI with 100 μg PPD/ml was nearly the same as MI tested with leucocytes preincubated with 50 μg PPD/ml.

Leucocyte Migration Using Capillary Tube Technique.

The MI was assessed for 25 tuberculin-positive persons, using the capillary tube technique with the application of 100 μg PPD/ml culture medium. Slight tuberculin-induced migra-

tion inhibition was found in 7 cases only, whilst the leucocytes in all the other cases showed no tuberculin-induced migration inhibition. These investigations were carried out in the usual manner, with horse serum in the culture medium.

In few cases, showing pronounced tuberculin-induced migration inhibition using agarose plate technique, the leucocytes were preincubated with PPD for $\frac{1}{2}$ hour and then set up with the capillary tube technique. With this modification of the capillary tube method, no tuberculin-induced migration inhibition was found either.

In 8 cases, the migration was tested also with fetal calf serum in the culture medium. In no case was tuberculin-induced migration inhibition found.

DISCUSSION

The present investigation shows that an agarose plate technique is suitable for the demonstration of tuberculin-induced migration inhibition.

Agarose plate technique has several advantages over capillary tube technique.

Investigations with tuberculin-induced migration inhibition have shown that the agarose plate technique is more sensitive than the capillary tube method.

The agarose plate technique is very saving in cells as only 1.5×10^6 leucocytes are used per culture, whereas with the capillary tube technique as used in the design of *Søborg & Bendixen*, about 9×10^6 leucocytes per culture are used. In the present investigation, there was no need for further saving in cells, but the agarose plate technique described can, supposedly, be adapted for cultures with a far lower number of cells.

When the agarose plate technique is used for the investigation of leucocytes preincubated with tuberculin, the method is very economical in tuberculin. This can be a great advantage when antigens are difficult to obtain.

When the agarose plates are prepared, the method is easier and quicker than the capillary tube method.

Investigation with Agarose Medium Containing Fetal Calf Serum.

Leucocytes from tuberculin-positive persons without the addition of PPD, cultured in agarose medium containing PPD showed none, or only slight tuberculin-induced migration inhibition, but if the same leucocytes were preincubated with PPD and placed in medium that did not contain PPD, the migration inhibition was seen clearly.

The reason for this difference in the migration inhibition between leucocytes preincubated with PPD and non-preincubated leucocytes placed in agarose medium containing PPD, is not known. Preincubating with PPD makes it possible for the high concentration of cells in the leucocyte suspension to have a more effective antigen-induced cell interaction in the preincubation time than the interaction which cannot start before the migration itself, when PPD is found only in the agarose medium.

Another possibility is that the more pronounced migration inhibition of preincubated leucocytes, is due to the fact that tuberculin-induced immune reactions in this case, start earlier than when the leucocytes are first exposed to the tuberculin in the agarose medium.

A corresponding difference in the tuberculin-induced migration inhibition between leucocytes preincubated with PPD and non-preincubated leucocytes could not be demonstrated using the capillary tube technique.

In few investigations, leucocytes from tuberculin-negative persons were resuspended in supernatants from leucocyte suspensions (2.2×10^8 leucocytes per ml) from tuberculin-positive persons. The tuberculin-negative leucocytes resuspended in supernatant from tuberculin-positive leucocyte suspensions preincubated for $\frac{1}{2}$ hour or 3 hours with PPD, showed migration areas just as large as if they were resuspended in

supernatant from the same tuberculin-positive leucocytes preincubated for $\frac{1}{2}$ hour or 3 hours with phosphate buffer. Any free migration inhibitory factor consequently was not demonstrated at this moment, where the leucocytes were placed on the agarose plate.

When the leucocytes were preincubated for $\frac{1}{2}$ hour with $50 \mu\text{g}$ PPD/ml, MI showed clearly a difference between a tuberculin-negative and a tuberculin-positive group, although there was considerable overlapping between the two groups. If the lowest limit for the tuberculin-negative persons is set to the mean value $-2 \times \text{SD}$, the limit will be 0.70. 8 out of 16 Mantoux II positive and 6 out of 23 Mantoux I positive persons had MI over this limit.

The reason for this low lower limit for tuberculin-negative persons could be due to pronounced unspecific toxic effect of PPD in the concentration $50 \mu\text{g}/\text{ml}$. However, it is probable that the tuberculin-negative group defined as persons with negative reaction after an intradermal injection of $0.2 \mu\text{g}$ PPD has included a number of persons who would have shown tuberculin hypersensitivity if their cutaneous reaction had been examined after larger doses of PPD. All the persons examined were adults and about 75 per cent were over 40 years of age. In Denmark, most adults will have been earlier spontaneous tuberculin-positive or have been vaccinated with BCG. Some from the tuberculin-negative group in the present study stated also that earlier they had been vaccinated with BCG, and others thought that earlier they had appeared to be spontaneous Mantoux-positive. It is possible, therefore, that several persons from the tuberculin-negative group on account of a slight tuberculin hypersensitivity has shown weak tuberculin-induced migration inhibition, but that their tuberculin hypersensitivity has been so weak, that they have not shown positive reaction with an intradermal injection of $0.2 \mu\text{g}$ PPD.

Leucocytes from tuberculin-positive persons clearly showed also migration inhibition when they were preincubated with $25 \mu\text{g}$ PPD/ml. Tuberculin-negative persons, however, had a