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DOSE-EFFECT RELATIONSHIP
BETWEEN TUBERCULIN CONCENTRATION
AND TUBERCULIN-INDUCED MIGRATION
INHIBITION OF HUMAN PERIPHERAL
BLOOD LEUKOCYTES DEMONSTRATED BY
AGAROSE MIGRATION TECHNIQUE

By

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In studies on antigen-induced inhibition of in vitro cell migration, a dose-effect relationship has been demonstrated both by explant and by capillary tube migration technique (1, 5, 8, 9). In some of these studies (8, 9) it was observed that migration of cells from animals or man with cellular immunity was stimulated by low concentrations of the specific antigen, even if migration inhibition was induced by higher concentrations.

The purpose of the present experiments was to study the effect of varying doses of tuberculin on migration of human peripheral blood leukocytes (PBL) using the agarose migration technique described by Clausen (2).

MATERIALS AND METHODS

The study comprised 40 persons. Patients with diseases which are supposed to influence cellular immunity and patients under treatment with glucocorticoids were not included in the study.

Skin Tests

Migration studies were carried out before skin testing. Mantoux-positive persons are defined as those who develop an induration of 5 mm or more 72 hr after intracutaneous injection of 0.1 ml tuberculin solution (Statens Serum-institut, Copenhagen, Denmark) containing 0.02 μ g purified protein derivative of tuberculin (PPD) (Mantoux I test) or 0.2 μ g PPD (Mantoux II test). None of the persons showed signs of active tuberculosis. Some had been vaccinated with BCG, others were spontaneous tuberculin-positive. Mantoux-negative persons are defined as those who had a negative intracutaneous reaction to both concentrations of PPD.

Tissue Culture Medium

Hepes-buffered TC medium was prepared from 100 ml TC 199, 10X (Difco Laboratories, Detroit, Mich., U.S.A.), 6.2 g Hepes (N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid, Sigma Chemical Company, St. Louis, Mo., U.S.A.), 500,000 i.u. penicillin, 500 mg streptomycin, and distilled water added to 1000 ml. The pH of the medium was adjusted to 7.40 with 5 N NaOH. The medium was sterilized by filtration through Millipore filters, 0.22 μ pore size.

Agarose Medium

The gel medium, which was prepared as previously described (2), was composed of 1 per cent agarose (Litex, Glostrup, Denmark), 10 per cent horse serum (Statens Seruminstitut), penicillin 66 i.u. per ml and 66 μ g streptomycin per ml (TC penicillin-streptomycin, desiccated, Difco Laboratories). Sodium bicarbonate (TC sodium bicarbonate solution 10 per cent, Difco Laboratories) was added, so that the pH of the medium after incubation in 2 per cent CO₂ in air was between 7.2 and 7.4. Five milliliters of agarose-serum-TC medium 199 was poured into disposable 50-mm plastic Petri dishes (Millipore Filter Corp., Bedford, Mass., U.S.A.). When the gel had formed, wells with a diameter of 2.3 mm were punched out in the agarose medium.

Migration Studies

Migration of leukocytes from cell suspensions containing varying amounts of PPD. Heparinized venous blood was mixed with 5 per cent dextran 250 (dextran 250, m.w. 250,000, Pharmacia, Uppsala, Sweden) in saline solution in the proportion 4:1. After sedimentation for 1 hr at 37° C, the cells of the leukocyte-rich plasma were washed three times in Hanks' balanced salt solution and re-suspended in Hepes-buffered TC 199 with 10 per cent horse serum to give a cell concentration of 2.2×10^6 /ml. This PBL suspension was divided into 90- μ l portions to which was added 10 μ l phosphate buffer containing varying amounts of PPD. As control, 10 μ l phosphate buffer without PPD was added to one of the PBL portions. The PPD concentration of the cell suspensions was 400, 300,

200, 100, 50, 25, 12.5, 6.3, 3.1 or 1.6 $\mu\text{g/ml}$; but each person was tested only with selected PPD concentrations. After incubation for $\frac{1}{2}$ hr at 37°C , 7- μl portions from the different PBL suspensions were placed in wells in agarose medium which contained neither PPD nor phosphate buffer. Six to eight cultures were made from each PBL suspension. The agarose plates were incubated at 37°C in 2 per cent CO_2 in air saturated with water vapor. After 20 hr the migration areas were studied under a projection microscope and measured by planimetry. A migration index (MI) was calculated as the ratio between the average area of PPD-stimulated cultures and that of control cultures. MI is marked with a figure indicating the PPD concentration used.

Migration of leukocytes washed after preincubation with PPD. The leukocytes were incubated with 100 μg PPD/ml. After incubation for $\frac{1}{2}$ hr, half of the PPD-containing and half of the control PBL suspension were placed in agarose medium as described above. The other halves were washed three times in Hanks' balanced salt solution and then resuspended in Hepes-buffered TC 199 with 10 per cent horse serum to give a final cell concentration of $2.1 \times 10^5/\text{ml}$. Neither PPD nor phosphate buffer was added to the resuspended cells. After further incubation for $\frac{1}{2}$ hr, 7- μl portions from both PBL suspensions were placed in agarose medium. Migration areas were measured after 4 hr (4 experiments) or 20 hr (7 experiments).

RESULTS

The data in Table 1 show the relationship between PPD concentration and PPD-induced migration inhibition. The experiments are arranged in order of decreasing MI_{100} . In experiment 8, MI_{200} and MI_{400} were the same, but in the other experiments 2 to 10, increasing PPD concentration induced a steadily increasing migration inhibition. In contrast, two or more of the highest PPD concentrations used in the experiments 11, 12, 13, and 15 to 29 induced about the same migration inhibition. This means that among the persons with the most PPD-sensitive PBL a maximum migration inhibition was obtained. It is seen that with increasing PPD-sensitivity (decreasing MI_{100}) the lowest PPD concentration, able to induce maximum inhibition, tend to fall.

In experiments 2 to 29 there was a good correlation between MI_{100} and $\text{MI}_{12.5}$, MI_{25} , MI_{50} , MI_{200} , and MI_{400} ($r = 0.88, 0.97, 0.97, 0.96$, and 0.92 , respectively; $p < 0.001$ applying to all PPD concentrations).

In 14 experiments (2, 4, 5, 8, 9, 10, 12 to 16, 22, 26, and

TABLE

Dose-Effect Relationship between PPD Concentration and PPD-

Exp. No.	Cutaneous reaction (mm) after PPD (μ g)		Control migration area (mm ²)
	0.02	0.2	
1	0	0	66.9
2	6		45.4
3	0	9	35.3
4	15		32.3
5	14		41.1
6			32.8
7	13		33.8
8	0	11	38.1
9	20		41.2
10	0	10	37.1
11	7		60.6
12	0	11	24.6
13	22		63.6
14	0	12	15.8
15	13		43.3
16	17		49.6
17			35.8
18	17		44.0
19			59.6
20	19		38.1
21	20		44.6
22	14		37.7
23	24		40.7
24	8		37.0
25	14		19.1
26	12		37.0
27	8		28.9
28			38.5
29	0	7	34.0

27), the leukocytes were tested with PPD concentrations of 25, 50, 100, 200 as well as 400 μ g/ml. Mean MI and standard deviations (SD) at these different PPD concentrations were 0.68 (SD 0.18), 0.61 (SD 0.17), 0.53 (SD 0.17), 0.48 (SD 0.16), and 0.42 (SD 0.13), respectively. The SD at

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Induced Migration Inhibition of Human Peripheral Blood Leukocytes

Migration indices at various PPD concentrations ($\mu\text{g/ml}$)								
1.6	3.1	6.3	12.5	25	50	100	200	400
					0.98	0.97	0.93	0.92
		0.97	0.91	0.90	0.86	0.79	0.75	0.67
1.01	1.00	0.99	1.07	1.01	0.97	0.77		
		0.95	0.94	0.92	0.87	0.77	0.77	0.66
		0.93	0.87	0.89	0.79	0.73	0.65	0.51
0.95	0.92	0.92	0.93	0.87	0.83	0.68		
1.00	0.97	0.97	0.95	0.90	0.75	0.68		
		0.93	0.86	0.83	0.70	0.65	0.48	0.48
		0.83	0.79	0.76	0.72	0.63	0.55	0.44
		0.87	0.78	0.72	0.64	0.58	0.50	0.38
					0.60	0.55	0.54	0.52
				0.67	0.61	0.55	0.54	0.49
		0.91	0.83	0.74	0.63	0.49	0.39	0.35
		0.84	0.86	0.70	0.58	0.47	0.49	0.36
		0.82	0.71	0.52	0.45	0.42	0.39	0.36
		0.92	0.81	0.64	0.49	0.42	0.39	0.35
					0.38	0.41	0.37	0.34
	0.60	0.53	0.50	0.47	0.47	0.41		
					0.41	0.40	0.39	0.39
0.93	0.87	0.72	0.59	0.49	0.39	0.38		
					0.39	0.34	0.34	0.32
		0.78	0.61	0.49	0.42	0.34	0.34	0.33
0.95	0.94	0.81	0.58	0.45	0.32	0.33		
0.96	0.92		0.69		0.32	0.31	0.33	
0.90	0.79	0.62	0.51	0.42	0.35	0.31		
		0.71	0.53	0.46	0.41	0.30	0.27	0.27
		0.63	0.49	0.34	0.31	0.27	0.26	0.23
0.37	0.33	0.29	0.26	0.29	0.25			
	0.46	0.29	0.22	0.24	0.22			

PPD concentrations of 25, 50, 100, and 200 $\mu\text{g/ml}$ were practically the same, but even that at 400 $\mu\text{g PPD/ml}$ did not differ significantly from the other SD ($p > 0.1$, by F-test).

PPD-induced migration stimulation was not observed in the present study.

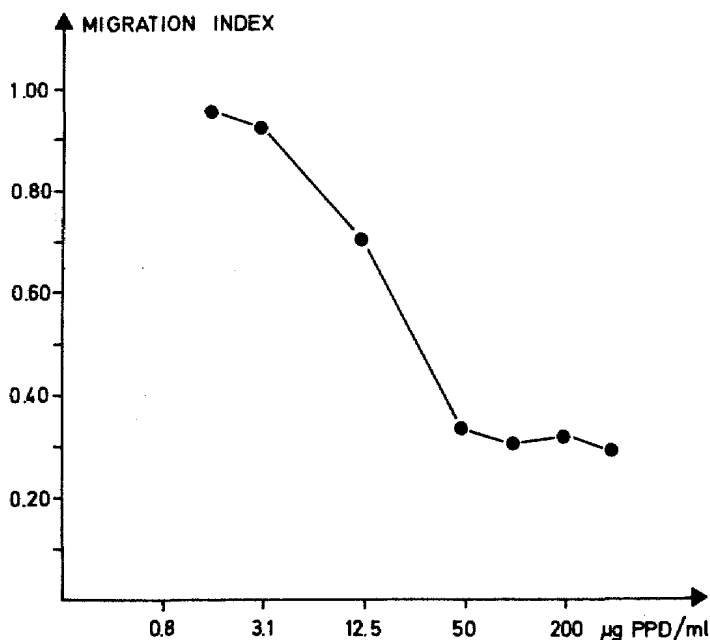


Fig. 1.

Dose-effect relationship between PPD concentration and PPD-induced migration inhibition of peripheral blood leukocytes from a Mantoux-positive person. After preincubation for $\frac{1}{2}$ hr with PPD doses ranging from 1.6 to 300 $\mu\text{g/ml}$, the leukocytes were placed in wells in agarose medium.

A typical sigmoid dose-effect curve is shown in Fig. 1. In that experiment a PPD concentration of 50 $\mu\text{g/ml}$ induced a maximum migration inhibition which was not increased by higher concentrations.

Fig. 2 shows experiments (13, 15, 16, 20, and 22 to 27 from Table 1), in which a greater part of the dose-effect curve could be drawn.

25 of the persons were skin tested, 18 of these had a positive Mantoux I reaction. Within the Mantoux I-positive group there was no correlation between the size of the skin

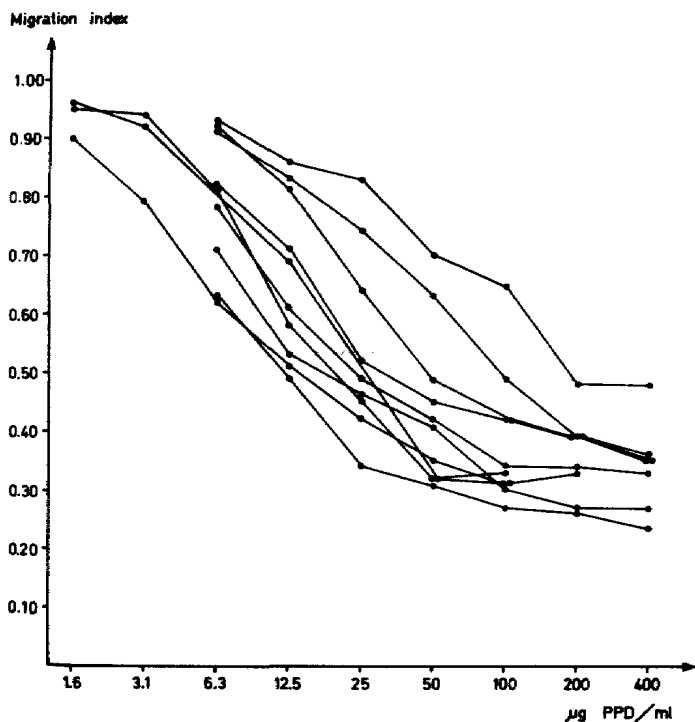


Fig. 2.

Dose-effect curves of PPD-induced migration inhibition of peripheral blood leukocytes from 10 persons. After preincubation for $\frac{1}{2}$ hr with varying doses of PPD, the leukocytes were placed in wells in agarose medium.

reaction and the $MI_{12.5}$, MI_{25} , MI_{50} , MI_{100} , MI_{200} or MI_{400} ($r = -0.06$, -0.08 , -0.17 , -0.18 , -0.07 , and -0.14 , respectively; $p > 0.1$ applying to all PPD concentrations).

Migration of PPD-sensitive PBL was inhibited, even if the cells were washed three times after incubation for $\frac{1}{2}$ hr with PPD (Table 2). In 8 out of 11 experiments, MI of PPD-preincubated, washed PBL was higher than that of PBL from the same person tested by the usual leukocyte migration agar-

TABLE 2

Migration Inhibition of Leukocytes Washed after Preincubation with PPD.

Exp. No.	Leukocytes incubated with 100 µg PPD/ml for ½ hr and then treated in different ways		Leukocytes washed, resuspended in TC medium without PPD, incubated further for ½ hr and then placed in agarose medium	
	PPD-containing leukocyte suspension placed in agarose medium	Migration indices (MI)	Migration areas (mm ²) of control cultures	Migration indices (MI)
MI measured after 4 hr				
1	24.2	0.63	21.0	0.80
2	31.9	0.58	33.9	0.65
3	26.2	0.51	23.5	0.51
4	37.7	0.31	28.7	0.36
MI measured after 20 hr				
5	35.1	0.65	25.7	0.63
6	39.8	0.57	20.7	0.56
7	44.9	0.53	33.9	0.56
8	35.8	0.46	24.3	0.49
9	49.9	0.42	23.6	0.78
10	41.4	0.27	26.1	0.46
11	23.5	0.27	14.3	0.35

ose test (LMAT). The mean MI of washed PBL was 0.09 higher than that of the same unwashed cells. This difference was significant ($p < 0.05$, t-test for paired comparisons).

DISCUSSION

The present study shows a typical dose-effect relationship between PPD concentration and PPD-induced migration inhibition of PBL from Mantoux-positive persons tested by direct LMAT.

In studies of cell migration from guinea pig spleen explants, Švejar et al. (8) observed that migration of cells from tuberculin-sensitive animals was stimulated by low doses of PPD

(0.1 and 0.5 $\mu\text{g/ml}$), but inhibited by higher doses (10 and 50 $\mu\text{g/ml}$). In another study, Švejcar & Johanovský (7) showed that the high doses of PPD stimulated cell migration during the first hours of culture, even if the final 18-hr measurement showed a pronounced migration inhibition.

In a capillary tube migration study on brucella hypersensitivity in man, Søborg (9) observed antigen-induced migration stimulation, especially when PBL from persons with weak hypersensitivity were stimulated by low doses of antigen.

In the present study no migration stimulation was observed, not even when PBL with low degree of PPD-sensitivity (high MI_{100}) were tested with a PPD concentration as low as 1.6 $\mu\text{g/ml}$. Neither was early migration stimulation observed in a previous study (4) in which it was shown that MI_{100} of a specifically reactive culture measured after 2 and 4 hr was significantly lower than the 20-hr MI. However, it is possible that migration stimulation might have occurred if PPD concentrations lower than 1.6 $\mu\text{g/ml}$ had been used. Another possibility is that agarose migration technique is less sensitive than explant and capillary tube migration techniques with regard to demonstrate a slight migration stimulation.

False negative results of LMAT has been observed even at a PPD concentration of 100 $\mu\text{g/ml}$ (2, 3, 4). The present study indicates that one way to avoid false negative results is to use PPD concentrations higher than 100 $\mu\text{g/ml}$. However, the normal range of MI_{200} and MI_{400} of Mantoux-negative persons has not yet been investigated. Only one Mantoux-negative person was included in the present study; but the observation that MI_{200} and MI_{400} were about the same as MI_{100} in experiments 8, 11, 12, 13, 15, 16, 17, 19, 21, 22, 25, and 26 indicates that PPD concentrations of 200 and 400 $\mu\text{g/ml}$ did not influence leukocytes in a non-specific toxic way. This finding is in agreement with the study of Federlin et al. (6) who by use of a microcapillary tube technique observed that a PPD concentration of 300 $\mu\text{g/ml}$ inhibited migration of PBL from Mantoux-positive, but not PBL from Mantoux-negative persons. Therefore, the falling MI using 200 and

400 μg PPD/ml in experiments 2, 4, 5, 9, 10, and 14 probably reflected an increasing specific effect. Another way to avoid false negative results is to measure the migration inhibition after only 4 hr of culture (4).

The size of the Mantoux I skin reaction did not correlate with MI_{100} . This is in agreement with two previous studies (3, 4). Nor did the skin reaction in the present study correlate with MI using any of the other PPD concentrations.

When LMAT is used as an in vitro screening test of tuberculin hypersensitivity it is desirable to do the test with only one or two PPD concentrations. If the most important point is to distinguish between a Mantoux-positive and a Mantoux-negative person it is of decisive importance to avoid false negative results. This may be obtained by use of a high PPD concentration. But if a quantitation of the migration inhibition reaction is the most important point, Fig. 2 suggests that MI at maximum inhibition will differ less than MI induced by lower PPD concentrations. However, in 14 experiments in which the migration inhibition was tested at both 25, 50, 100, 200, and 400 μg PPD/ml, the deviation of MI values around the mean was about the same at each PPD concentration.

On the other hand, if LMAT is used to evaluate, as much as possible, the specific reactivity of the lymphocytes, it is advisable to estimate the whole dose-effect curve and the time course of migration of PPD-stimulated as well as control cultures. A simple way to follow the time course of migration has, recently, been described (4).

A theoretical disadvantage of LMAT is the very high PPD concentration which has to be used. The PPD concentrations used in LMAT were also considerably higher than those used by Sørensen et al. (10) in a study on blast transformation of human lymphocytes. They found that the optimal concentration of PPD varied between 0.2 and 1.5 $\mu\text{g}/\text{ml}$. The reason for this difference as to optimal concentration of PPD between the LMAT and the lymphocyte transformation test may be that release and/or production of migration inhibition factor (MIF) from sensitive lymphocytes requires a higher

PPD concentration than that necessary to induce transformation of the lymphocytes. But the reason may also be related to the different time course of the immune response measured by the two *in vitro* tests. In the lymphocyte transformation test, DNA synthesis after 2 days of culture is very low, but then increases during the following days (10). Therefore, in blast transformation experiments a lag of response is unimportant. But in LMAT, MIF release during the first 4 hours of culture is quite decisive for the final migration inhibition. Partly because about three quarters of the cell migration, measured by area, occurs during that period (4) and partly because the MIF-sensitivity of the migrating cells probably decreases during the later part of the culture period (4). Such an early MIF release and/or production is obtained only by use of a high antigen concentration.

When PPD-containing PBL suspension is placed in agarose medium without PPD, the concentration of PPD around the cells must fall. Nevertheless, no further migration inhibition was obtained even though PPD was added to the agarose medium so that the PPD concentration in agarose medium and PBL suspension was the same (Clausen, unpublished experiments). In accordance with this, the present study shows that migration of PPD-sensitive PBL was inhibited even if the cells after incubation with PPD were washed three times before being placed in agarose medium. This corresponds also with a previous observation (3) that lymphocytes from Mantoux-positive persons were stimulated by preincubation with PPD for 2½ hr and then continued to produce MIF, even if the cells were washed and cultured in PPD-free TC medium. The lower degree of migration inhibition of washed cells in the present study may be caused by removal of PPD, but the reason may also be that the smaller migration areas, caused by the additional washings or the longer time from the blood was taken until the leukocytes were placed in agarose medium, imply a lower MIF-sensitivity of the test. With regard to PPD it is unnecessary to wash the cells after preincubation with PPD, because even high doses of PPD were without

non-specific toxic effects; but regarding other more toxic antigens it may be preferable to wash the cells after a preincubation period and thereby shorten the time in which the leukocytes are exposed to non-specific toxic effects.

SUMMARY

A dose-effect relationship between PPD concentration and PPD-induced migration inhibition was demonstrated. In about two thirds of the experiments a maximum migration inhibition was obtained. No PPD-induced migration stimulation was observed.

If PPD-sensitive PBL were incubated with PPD for $\frac{1}{2}$ hr and then washed carefully before being placed in agarose medium, the cell migration was still inhibited. Such a stimulation procedure may be preferable when antigens with strong toxic effects are used in migration studies.

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