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TUBERCULIN-INDUCED MIGRATION INHIBITION OF HUMAN PERIPHERAL BLOOD LEUKOCYTES AFTER 2, 4, AND 20 HOURS IN AGAROSE CULTURE

By

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The direct leukocyte migration agarose test (LMAT) described by Clausen (3) is a sensitive in vitro method for demonstration of tuberculin hypersensitivity. Nevertheless, false negative results are seen (3, 5). This may be because the leukocyte migration is uninhibited during the whole 20-hr culture period, but another possibility is that the leukocytes are inhibited only in the first few hours and thereafter migrate uninhibited, so that the migration index increases during the later culture period. Using the capillary tube technique, such a leukocyte escape from an early migration inhibition has been observed in animal (7, 8, 9) as well as in human experiments (6).

The purpose of the present study was to examine whether human peripheral blood leukocytes (PBL) in LMAT may also escape, more or less, an early tuberculin-induced migration inhibition.

MATERIAL AND METHODS

The study comprised 42 persons. Patients with diseases which are supposed to influence cellular immunity, and patients under treatment with glucocorticoids or cytostatics were not included in the study.

Skin Tests

A Mantoux test was made in all persons with migration index (MI) greater than 0.70 after a culture period of 4 or 20 hr in LMAT. Five of 28 persons with MI less than 0.70 were skin tested too. 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 (State Serum Institute, 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). Mantoux-negative persons are defined as those who had a negative intracutaneous reaction to both concentrations of PPD.

All migration studies were carried out before skin testing.

Agarose Medium

The gel medium, which was prepared as previously described (3), was composed of 1 per cent agarose (Litex, Glostrup, Denmark), 10 per cent horse serum (State Serum Institute), 66 i.u. penicillin and 66 μ g streptomycin (TC penicillin-streptomycin, desiccated, Difco Laboratories, Detroit, Mich., U.S.A.) in TC 199 (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.

Tissue Culture Medium

Hepes-buffered TC medium was prepared from 100 ml TC 199, 10 X (Difco Laboratories), 6.2 g Hepes (N-2-hydroxyethylpiperazine-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.

Culture Supernatants Containing Migration Inhibition Factor (MIF)

Cell-free supernatants with migration inhibitory effect were prepared from 2-day cultures of PPD-stimulated human mononuclear leukocyte cultures as previously described (5). After incubation with PPD or phosphate buffer for 2½ hr, the mononuclear leukocytes were washed three times and cultured in Hepes-buffered TC 199 with 10 per cent horse serum. Therefore, these supernatants did not contain PPD.

Direct PPD-Induced Migration Inhibition

Suspensions of PBL in Hepes-buffered TC 199 with 10 per cent horse serum were prepared from heparinized venous blood as previously described (3). Two types of experiments were carried out:

(a) *Migration inhibition after varying time of agarose culture.* PPD in phosphate buffer (State Serum Institute) was added to half of the PBL suspension to a final concentration of $100\text{ }\mu\text{g PPD/ml}$. As control, the same amount of phosphate buffer without PPD was added to the other half. The final cell concentration was $2.1 \times 10^8/\text{ml}$. After incubation for $\frac{1}{2}$ hr at 37°C , $7\text{-}\mu\text{l}$ portions from PPD-containing as well as control leukocyte suspension were placed in wells in agarose medium, which contained neither PPD nor phosphate buffer. The agarose cultures were incubated at 37°C in 2 per cent CO_2 in air saturated with water vapor. Migration areas were studied under a projection microscope and measured by planimetry after 4 hr in one group of agarose cultures and after 20 hr in another group. In 26 of 42 experiments migration areas were measured after 2 hr in a third group of cultures.

MI was calculated as the ratio between the average area of six PPD-stimulated cultures and that of six control cultures. For a comparison between leukocyte migration of PPD-stimulated and corresponding control cultures during the 4 to 20 hr culture period, increase of migration area during that period may be misleading, because the 4-hr migration area of the PPD-stimulated culture from a Mantoux-positive person nearly always will be smaller than that of the corresponding control culture. Consequently, an identical increase of migration radius (mm) of stimulated and control culture implies that the increase of migration area (mm^2) of the stimulated culture becomes smaller than that of the control culture. Therefore, since it is not reasonable to consider this as an expression of migration inhibition, a migration distance was used for such comparisons. The migration distance for some culture period was calculated as the radial distance between the peripheral border of the migration area at the beginning and that at the end of the culture period.

(b) *4-hr MI of leukocytes tested at various times after their preparation.* Part of freshly prepared PBL from eight Mantoux-positive persons were mixed with PPD or phosphate buffer as described above. After incubation for $\frac{1}{2}$ hr, $7\text{-}\mu\text{l}$ portions were placed in wells in agarose medium. The cell concentration of the remaining part of the PBL suspension was adjusted to $0.7 \times 10^8/\text{ml}$ by adding Hepes-buffered TC 199 with 10 per cent horse serum. In three experiments these PBL suspensions were divided into three equal parts. After incubation at 37°C for 6 hr (all experiments), 12 hr (three experiments), and 18 hr (three experiments) the cell concentration was readjusted to $2.1 \times 10^8/\text{ml}$ by centrifugation and removal of the surplus of culture medium. PPD or phosphate buffer was then added as described above and after further incubation for $\frac{1}{2}$ hr, $7\text{-}\mu\text{l}$ portions were placed in agarose medium. All migration areas were measured after 4 hr of culture.

Migration Inhibition of Polymorphonuclear Leukocytes (PMN) by MIF-Containing Culture Supernatants

Purified PMN were prepared by the Isopaque-Ficoll centrifugation procedure (1) as previously described (2, 4). The PMN were divided into two equal parts which were resuspended in cell-free culture media from control and PPD-stimulated cultures, respectively, to give a final cell concentration of 1.4×10^8 /ml. After incubation for 1 hr, 7- μ l portions were placed in agarose medium. The cells in the remaining parts of the PMN suspensions were washed three times in Hanks balanced salt solution and resuspended in fresh Hepes-buffered TC 199 with 10 per cent horse serum to give the same cell concentration as before the washing. Again 7- μ l portions were placed in agarose medium. Thereafter the cell concentration of the PMN suspensions were adjusted to 0.5×10^8 /ml by adding more Hepes-buffered TC 199 with 10 per cent horse serum. After further incubation for 4 hr the PMN concentration was readjusted to 1.4×10^8 /ml by removing the surplus of TC medium, and then 7- μ l portions were placed in agarose medium. Migration areas were measured after 4 hr. MI was calculated as the ratio between the average area of six cultures of PMN suspended in supernatant from PPD-stimulated cultures and that of six cultures suspended in the corresponding control supernatant.

RESULTS

Time Course of Leukocyte Migration

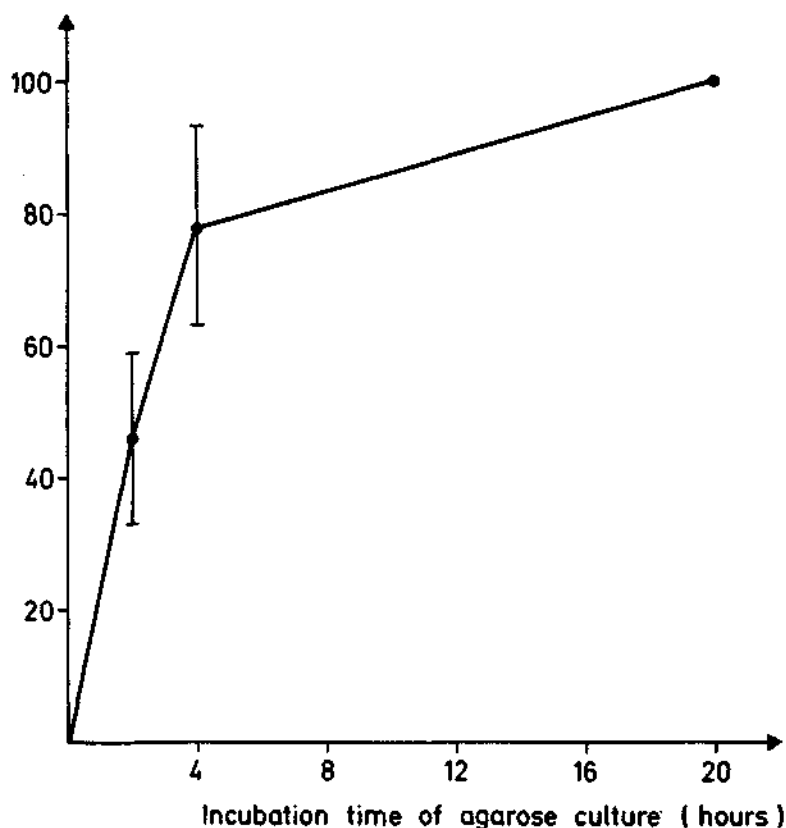
Fig. 1 shows that the mean migration area of unstimulated PBL after 2 and 4 hr of culture was 46 (SD 13) per cent and 78 (SD 15) per cent, respectively, of the 20-hr area. If the migration was expressed by migration distance the corresponding figures were 59 (SD 12) per cent and 84 (SD 11) per cent.

The 4-hr migration areas looked like the 20-hr areas, all with a clear-cut peripheral border. The border of the 2-hr migration areas, however, was sometimes a little blurred.

Relation between Culture Time and PPD-Induced Migration Inhibition

Table 1 shows MI after 2, 4, and 20 hr of culture. In experiments 3 to 42, the 4-hr MI was in average 0.08 lower than the 20-hr MI. This difference was significant ($p < 0.001$, t-test for paired comparisons). After 2 hr the MI was

Percentage migration

*Fig. 1.*

Time course of leukocyte migration using agarose migration technique. Non-stimulated human peripheral leukocytes were placed in wells in agarose medium. Migration areas after 2 and 4 hr are expressed as per cent of the 20-hr area. Vertical lines with bars indicate ± 1 SD.

in average 0.06 lower than that after 4 hr, but this difference was not significant ($p > 0.05$). In these experiments there was a good correlation between the MI after 4 and that after 20 hr ($r = 0.78$, $p < 0.001$). In experiments 3 to 42 the average migration distance of PPD stimulated cultures was 0.51 (SD 0.31) mm during the 4 to 20 hr culture period and that of control cultures was 0.47 (SD 0.33) mm. In 20 of these experiments the PPD-stimulated culture had a longer migration distance than the corresponding control culture during that period.

TABLE I

Time Course of Migration and Migration Inhibition of Control and PPD-Stimulated Agarose Cultures of Human Peripheral Blood Leukocytes.

Exp. No.	Cutaneous reaction in mm after PPD (μ g)		Migration areas (mm ²) of control cultures at varying culture time			Migration indices after varying time of culture		
	0.02	0.2	2 hr	4 hr	20 hr	2 hr	4 hr	20 hr
1	0	0		46.7	64.7		0.89	0.97
2	0	0	20.4	27.2	34.5	0.87	1.01	0.94
3	6		16.9	42.5	64.4	0.66	0.92	0.86
4	6			41.8	49.7		0.45	0.83
5	8			46.6	52.5		0.70	0.83
6	6			27.1	37.8		0.78	0.82
7	9		13.7	27.4	57.5	0.24	0.68	0.81
8	8			24.3	28.3		0.64	0.80
9	7		27.1	31.8	48.5	0.71	0.72	0.77
10	0	9	19.3	40.8	63.3	0.94	0.84	0.76
11	20			39.1	43.0		0.62	0.73
12	8			29.5	38.8		0.83	0.72
13	11			39.5	70.5		0.79	0.71
14			17.7	35.4	48.3	0.81	0.66	0.69
15	0	11		37.7	53.5		0.47	0.69
16	9			34.7	53.6		0.82	0.65
17			11.6	37.1	47.6	0.30	0.69	0.64
18	6			29.1	29.8		0.39	0.64
19			18.1	33.2	31.8	0.48	0.65	0.64
20	0	8		27.1	31.7		0.27	0.57
21			18.6	36.2	66.3	0.70	0.59	0.56
22			15.7	24.8	36.1	0.41	0.68	0.56
23			18.6	32.0	43.9	0.51	0.30	0.55
24			15.9	21.2	25.3	0.26	0.36	0.53
25			24.1	36.8	54.1	0.33	0.49	0.50
26			22.9	35.6	36.9	0.32	0.32	0.51
27			21.1	40.1	49.4	0.37	0.32	0.49
28	6			14.8	27.9		0.26	0.48
29			19.8	29.3	40.1	0.59	0.48	0.48
30			24.3	45.1	46.4	0.42	0.38	0.46

TABLE 1 (continued)

Exp. No.	Cutaneous reaction in mm after PPD (μ g)		Migration areas (mm^2) of control cultures at varying culture time			Migration indices after varying time of culture		
	0.02	0.2	2 hr	4 hr	20 hr	2 hr	4 hr	20 hr
31			19.1	33.0	52.9	0.29	0.32	0.46
32	0	7		29.9	29.3		0.33	0.44
33				32.3	37.7		0.25	0.44
34			19.8	30.4	42.8	0.26	0.19	0.43
35				23.9	20.9		0.23	0.43
36			17.8	29.6	47.4	0.38	0.32	0.40
37			23.9	40.6	36.2	0.26	0.29	0.39
38			18.3	18.4	24.9	0.41	0.49	0.38
39			28.1	47.4	68.9	0.24	0.24	0.37
40			22.9	36.5	44.2	0.27	0.39	0.32
41			22.3	35.4	44.3	0.29	0.40	0.32
42			18.4	23.3	32.3	0.13	0.27	0.29

Relation between Positive Mantoux Reaction and PPD-Induced Migration Inhibition

In the Mantoux I-positive group (Table 1), no significant correlation was found between the size of the intracutaneous tuberculin reaction and the MI, neither the 4-hr MI ($r = 0.12$, $p > 0.1$) nor the 20-hr MI ($r = -0.03$, $p > 0.1$).

Effect of Growing in vitro Age of PBL on PPD-Induced Migration Inhibition

Migration of PBL incubated at 37°C for 6 or 12 hr before being stimulated by PPD was still inhibited in LMAT, but the inhibition was greater if the PBL were tested just after their preparation (Table 2). The 4-hr MI of PBL incubated for 6 hr before being tested was in average 0.05 higher than that of the same PBL tested just after their preparation. This difference was significant ($p < 0.01$, t-test for paired comparisons). PBL incubated for 18 hr before being tested did not migrate.

TABLE 2

PPD-Induced Migration Inhibition of Leukocytes Tested at Various Times After Their Preparation.

Exp. No.	Migration parameters of agarose cultures					
	Leukocytes tested just after their preparation		Leukocytes incubated in TC medium for 6 hr before being tested		Leukocytes incubated in TC medium for 12 hr before being tested	
	4-hr areas (mm ²) of control cultures	4-hr indices	4-hr areas (mm ²) of control cultures	4-hr indices	4-hr areas (mm ²) of control cultures	4-hr indices
1	41.0	0.69	39.8	0.71		
2	24.2	0.63	25.4	0.71		
3	31.9	0.57	37.4	0.58		
4	26.2	0.51	20.2	0.55		
5	26.9	0.49	15.9	0.52	13.2	0.58
6	20.9	0.43	16.2	0.46	10.7	0.70
7	26.5	0.38	18.9	0.47	12.1	0.60
8	37.7	0.31	35.5	0.39		

TABLE 3

Effects of MIF-Containing Supernatants on Migration of Polymorphonuclear Leukocytes.

Exp. No.	Polymorphonuclear leukocytes incubated for 1 hr in MIF-containing supernatant and then treated in different ways					
	Without further treatment placed in agarose medium		Washed and resuspended in fresh TC medium			
			Placed immediately in agarose medium		Placed in agarose medium after 4 hr of incubation	
	4-hr migration areas (mm ²) of control cultures	4-hr migration indices	4-hr migration areas (mm ²) of control cultures	4-hr migration indices	4-hr migration areas (mm ²) of control cultures	4-hr migration indices
1	25.7	0.68	27.2	0.63	20.3	0.63
2	15.9	0.60	11.9	0.54	11.7	0.52
3	30.4	0.57	30.9	0.60	27.2	0.59
4	24.0	0.54	19.4	0.47	20.5	0.52
5	30.5	0.50	23.2	0.54	24.0	0.53
6	16.6	0.39	15.6	0.37	15.2	0.40

Migration Inhibition Induced by MIF-Containing Supernatants

Migration of PMN exposed to MIF by incubating the cells for 1 hr in MIF-containing supernatant was inhibited (Table 3), and the inhibition persisted unaltered if the cells were washed three times after the exposure to MIF. These washed PMN were inhibited to the same degree even if they were incubated for 4 hr in fresh TC medium before being placed in wells in agarose medium.

DISCUSSION

The reported data show that the migration rate of unstimulated PBL in LMAT was much higher during the first 4 hours than later on. Since PBL incubated for 6 hr before being tested had 4-hr control migration areas almost as large as those of PBL tested just after their preparation, the small migration during the 4 to 20 hr culture period cannot be explained by growing age of the cells. Probably, the high migration rate during the first few hours of agarose culture was due to the high number of leukocytes in the well at the onset of that period. This explanation is supported by the observation (Clausen, unpublished experiments) that the cell concentration of a leukocyte suspension, used for agarose cultures, was quite decisive for the size of the final 20-hr migration area. On the other hand, in the latter half of the culture period slowing of the migration rate was probably to some extent caused by growing age of the migrating cells, as PBL incubated for 12 hr before being placed in wells in agarose medium migrated considerably less than PBL placed in agarose medium just after the cell preparation.

PPD-induced migration inhibition of human PBL was significantly greater after 4 than after 20 hr. In previous migration studies (Clausen, unpublished experiments), the mean 20-hr MI of 14 Mantoux-negative persons was 0.92 (SD 0.05). In the present study four Mantoux-positive persons had 20-hr MI greater or equal to 0.82, the lower limit of the normal range (95 per cent confidence limits) of Mantoux-

negative persons. The four persons with false negative results after 20 hr of culture had 4-hr MI of 0.45, 0.70, 0.78, and 0.92. The person with the 4-hr MI of 0.92 had a 2-hr MI of 0.66. Probably, these values except the 4-hr MI of 0.92 reflected a specific PPD-induced migration inhibition. As the partial escape from an initial migration inhibition resulted in 10 per cent false negative 20-hr results, it seems advisable to measure the migration inhibition not only after 20 hr, but also after a shorter culture period. Measurement of migration areas after 2 hr was a little uncertain, partly because the areas were small (control cultures about 46 per cent of the 20-hr areas) and partly because the peripheral borders were less well-defined. After 4 hr of culture the migration areas were about three quarters of the 20-hr areas and the peripheral borders were always clear-cut. Therefore, a 4-hr measurement seems ideal.

In 20 experiments, in which the 4-hr MI showed a PPD-induced migration inhibition, the 4 to 20 hr migration distance of PPD-stimulated cultures was longer than that of corresponding control cultures. But a longer migration distance of a PPD-stimulated culture during the last 16 hr culture period cannot be taken as evidence of a migration stimulation during that period, since the higher density of cells in the small migration area of a 4-hr PPD-stimulated culture must inevitably result in a longer 4 to 20 hr migration distance than that of the corresponding control culture. PPD-induced migration stimulation is unproved, as long as the 20-hr area of the PPD-stimulated culture is less or equal to that of the control culture.

No correlation was demonstrated between the size of the Mantoux reaction and the direct PPD-induced migration inhibition, neither after 4 nor after 20 hr. Although it is assumed that the *in vivo* as well as the *in vitro* parameters of tuberculin hypersensitivity are caused by specific stimulation of PPD-sensitive lymphocytes, it is understandable that the very complex intracutaneous reaction did not correlate with the more simple migration inhibition reaction. One essential difference is that in LMAT the result is quite depending on MIF

release from lymphocytes during the first few hours of culture, whereas the Mantoux reaction is influenced, probably, by MIF production during all three days from the intradermal injection of PPD until the measurement of the reaction. This may explain why, in a previous study (5), the Mantoux reaction was correlated with the migration inhibitory effect of cell-free supernatants from 2-day cultures of PPD-stimulated human mononuclear leukocytes, but not with the 20-hr MI of direct LMAT.

As the result of direct LMAT is dependent on MIF release during the first few hours of culture, false negative results may appear if MIF release is delayed, but in the present study only experiment 10 of Table 1 indicated such a delayed MIF release.

When PPD-containing PBL suspension is placed in PPD-free agarose medium, the PPD concentration around the cells has to decrease. This, however, cannot explain the partial escape of PBL from an early migration inhibition, since it has been shown (Clausen, unpublished experiments) that PPD-induced migration inhibition was identical in agarose medium without PPD and agarose medium containing PPD in the same concentration as that used in the PBL suspension.

If PMN migrate faster than the MIF producing lymphocytes, the MIF concentration around the PMN has to decrease. However, in a previous study (3), PMN as well as mononuclear leukocytes were seen in the whole migration area. But even if PMN migrate away from MIF producing lymphocytes, the present study shows that migration of PMN, once exposed to MIF, was inhibited even if the cells were washed thoroughly before being cultured in agarose medium. This is probably caused by a strong binding of MIF to the cells or by an irreversible MIF-induced alteration of the cells.

On the other hand, the escape has presumably something to do with the growing age of the cells during the culture period. Escape is probably not caused by decreasing lymphocyte function. Lymphocytes remain viable for several days in leukocyte cultures, and in a previous study (5) it has been shown that

the migration inhibitory effect of cell-free supernatants from PPD-stimulated human mononuclear leukocyte cultures was greater after two and especially after three days of culture than after only one day.

PMN, in contrast to lymphocytes, are short-lived cells *in vivo* as well as under *in vitro* culture conditions. In consequence of this, although still being able to migrate, it is possible that PMN become unsensitive or less sensitive to MIF in the later part of the culture period. This hypothesis is consistent with the finding that migration of PBL incubated in TC medium for 6 and especially 12 hr before being tested by LMAT was less inhibited than if the cells were tested just after their preparation. Nevertheless, migration of PMN, washed after being exposed to MIF, was inhibited to the same degree just after the washing and 4 hr later; but if the washed PMN had been incubated for 6 or 12 hr before being cultured in agarose medium, the migration inhibition would, probably, have been less pronounced.

SUMMARY

Migration rate of human peripheral blood leukocytes in agarose culture was highest during the first few hours, and after 4 hr the migration areas of control cultures were about three quarters of the 20-hr areas. PPD-induced migration inhibition of PPD-sensitive leukocytes was greater after 4 than after 20 hr, and in half of the experiments the 4 to 20 hr migration distance of PPD-stimulated cultures was longer than that of the corresponding control cultures. In order to avoid false negative results, it is advisable to supplement the ordinary 20-hr measurement with one after 4 hr.

A falling concentration of MIF during the culture period cannot explain the escape phenomenon, since the migration of purified PMN, once exposed to MIF, was inhibited even if the cells were washed thoroughly before being cultured in agarose medium.

Leukocytes incubated for 6 and especially 12 hr before

being tested with PPD in the migration agarose test were inhibited less than if the same cells were tested just after their preparation. In consequence of this, it is proposed that the partial escape from early migration inhibition was caused by decreasing MIF-sensitivity of the migrating polymorphonuclear leukocytes during the later part of the culture period. Therefore, it is a great advantage by the agarose migration technique that most of the migration takes place during the first 4 hr of culture.

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