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COMPARISON BETWEEN CAPILLARY TUBE AND AGAROSE MIGRATION TECHNIQUE IN THE STUDY OF HUMAN PERIPHERAL BLOOD LEUKOCYTES

By

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The capillary tube migration technique introduced by Georg & Vaughan (9), modified by David et al. (6), and adapted for studies of human peripheral blood leukocytes (PBL) by Søborg & Bendixen (16) has, among other things, been used to demonstrate tuberculin-induced migration inhibition of PBL from Mantoux-positive persons (1, 8, 14, 15, 17). However, using this technique several groups have reported their inability or difficulty to demonstrate hypersensitivity to purified protein derivative of tuberculin (PPD) (2, 11, 12, 13, 15, 17). In contrast, the agarose migration technique described by Clausen (2) seems to be a very reliable technique for demonstrating PPD-induced migration inhibition of PBL from Mantoux-positive persons (2, 4, 5). In a previous study (2) it was planned to compare the leukocyte migration capillary tube test (LMCT) with the leukocyte migration agarose test (LMAT), but this was abandoned because PBL from Mantoux-positive persons only occasionally were inhibited in LMCT. Nevertheless, sometimes a migration inhibition in LMCT was observed during the first 6 hr of culture, but disappeared later on. Such an escape phenomenon has been described also using LMAT (5).

In the present study the escape from early PPD-induced migration inhibition in LMCT was studied, and the early migration inhibition was compared with that observed in LMAT. Furthermore, the capillary tube and the agarose migration techniques were compared concerning their ability to demonstrate migration inhibition factor (MIF) of cell-free supernatants from cultures of PPD-stimulated tuberculin-sensitive human mononuclear leukocytes or from mixed human lymphocyte cultures (MLC).

MATERIALS AND METHODS

The study comprised 73 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

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 (State Serum Institute, Copenhagen, Denmark) containing 0.02 μg PPD (Mantoux I test) or 0.2 μg PPD (Mantoux II test). 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 (State Serum Institute), 66 i.u. penicillin and 66 μg streptomycin (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 CO_2 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.

MIF-Containing Supernatants

Cell-free supernatants from MLC (the mixed culture and the two unmixed control cultures) or from PPD-stimulated and corresponding control cultures of human mononuclear leukocytes were prepared as previously described (3, 4).

Seven of the MIF-containing supernatants from PPD-stimulated cultures contained PPD (Sup-PPD, type I), because the mononuclear leukocytes were cultured in presence of PPD. In these experiments, PPD was added to the cell-free control supernatants so that the PPD concentration in MIF-containing and control supernatants was the same. Supernatants were prepared after one day of culture.

Seven other MIF-containing supernatants from PPD-stimulated cultures contained no or only extremely small amounts of PPD (Sup-PPD, type II), since the mononuclear leukocytes after incubation with PPD for 2½ hr were washed three times and then cultured in TC medium without PPD. These supernatants were prepared after one, two or three days of culture.

Direct PPD-Induced Migration Inhibition

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 resuspended in Hepes-buffered TC 199 with 10 per cent horse serum. PBL were tested by both capillary tube and agarose migration technique.

Capillary tube migration technique. Two different types of stimulation procedure were used.

In series A experiments the technique described by Søborg & Bendixen (16) was used. PBL suspension was drawn into eight siliconized glass capillary tubes (1.4 mm inner diameter). The capillaries were closed by heating in a gas flame. After centrifugation at 900 x G for 10 min, the capillaries were cut 1 mm below the cell-liquid interface and immediately placed in 1-ml culture chambers containing Hepes-buffered TC 199 with 10 per cent horse serum. The bottoms of the chambers were made of siliconized glass or polystyrene. PPD in phosphate buffer (State Serum Institute) was added to half of the chambers to give a concentration of 100 μg PPD/ml. As control, the same amount of phosphate buffer without PPD was added to the other half. The culture chambers were incubated at 37° C. After 4, 6, and 20 hr the migration areas were studied under a projection microscope, traced on paper and measured by cutting out and weighing. A migration index (MI) was calculated as the ratio between the average area of PPD-stimulated cultures and that of control cultures.

In series B experiments PPD in phosphate buffer was added to half of the PBL suspension to a final concentration of 100 μg PPD/ml. As control, the same amount of phosphate buffer without PPD was added to the other half. The final cell concentration was 2.1×10^8/ml. After incubation for ½ hr at 37° C, both PBL suspensions were drawn into capillary tubes which were treated as described above and placed in culture chambers with polystyrene bottoms. Neither PPD nor phosphate buffer was added to the TC medium in the culture chambers. Migration areas were measured after 4 and after 20 hr.

In four experiments, in which culture chambers with bottoms of polystyrene were used, PPD-induced migration inhibition was examined according to the experimental scedule for series A as well as that for series B.

Agarose migration technique. PPD-containing and corresponding control PBL suspension were prepared as described above (series B stimulation procedure of capillary tube migration technique). After incubation for ½ hr at 37° C, 7-μl portions were placed in wells in agarose medium which contained neither PPD nor phosphate buffer. Six cultures were made from both PBL suspensions. 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. MI was calculated as described above.

In 12 experiments, the PBL suspension was used for capillary tube (series B procedure) as well as agarose migration studies.

Inhibition of PBL Migration by Culture Supernatants

PBL prepared as described above were divided into two equal portions which were resuspended in the cell-free culture media from control and stimulated cultures, respectively, to a final cell concentration of 2.1×10^8/ml. After incubation for 1½ hr, the cells were studied using both capillary tube and agarose migration technique.

Culture supernatants containing PPD were tested on PBL which were not inhibited by 100 μg PPD/ml, neither in LMCT nor in LMAT.

Capillary tube migration technique. PBL suspended in control as well as in MIF-containing supernatant were drawn into capillary tubes which were placed in culture chambers with polystyrene bottoms. No supernatant was added to the Hepes-buffered TC 199 with 10 per cent horse serum in the culture chamber. Four cultures were prepared from both PBL suspensions. Migration areas were measured after 4 and after 20 hr of culture. The migration inhibitory effect of a supernatant from a stimulated culture was expressed by a MI which indicates the ratio between the average area of PBL suspended in supernatant from stimulated cultures and that of PBL suspended in supernatant from the corresponding control cultures.

Agarose migration technique. Six 7-μl portions from both PBL suspensions were placed in wells in agarose medium. Migration areas were measured after 20 hr. MI were calculated as described above.

RESULTS

Time Course of Migration of PBL in LMCT

Using capillary tube migration technique and culture chambers with glass bottoms (Table 1), the mean 4-hr MI of the Mantoux-negative group was 0.94 (SD 0.07) and that of the Mantoux-positive group 0.75 (SD 0.12). The corresponding 20-hr MI values were 0.94 (SD 0.07) and 0.98 (SD 0.08). The Mantoux-negative and the Mantoux-positive group differed significantly (by t-test) with regard to the 4-hr MI ($p < 0.001$), but not with regard to the 20-hr MI ($p > 0.1$). 4-hr MI of the Mantoux-positive persons was in average 0.05 lower than 6-hr MI and 0.23 lower than 20-hr MI. The difference between the 4-hr and the 20-hr MI was significant ($p < 0.001$, t-test for paired comparisons), but that between the 4-hr and the 6-hr MI was not ($p > 0.05$).

Fig. 1 shows that the mean migration area of unstimulated capillary tube leukocyte cultures after 4 and 6 hr was 27 (SD 7) per cent and 44 (SD 9) per cent, respectively, of the mean 20-hr area.

In five experiments (Table 1), PBL migration was studied both in chambers with glass bottoms and in chambers with polystyrene bottoms. Control migration areas on polystyrene were about twice as large as those on glass. 4-hr and 6-hr control areas expressed as per cent of 20-hr areas were about the same on glass and on polystyrene. PPD-induced migration inhibition on polystyrene was significantly more pronounced than that on glass. In culture chambers with bottoms of polystyrene the mean 4-hr MI was 0.28, the mean 6-hr MI 0.32, and the mean 20-hr MI 0.31 lower than the corresponding mean MI obtained in culture chambers with bottoms of glass. These differences were all significant ($p < 0.025$, $p < 0.001$, and $p < 0.001$, respectively, t-test for paired comparisons).

Migration areas of capillary tube cultures all had clear-cut peripheral borders after 4 and 6 hr of culture, but after 20 hr the borders were less well-defined, because the cell density gradually decreased over a rather long distance. Sometimes, a

TABLE 1

Time Course of Migration and Migration Inhibition of Control and PPD-Stimulated Capillary Tube Cultures of Human Peripheral Blood Leukocytes. Bottoms of Culture Chambers Were Made of Glass or Polystyrene.

Exp. No.	Cutaneous reaction in mm after PPD (μg)		Migration areas (mm^2) of control cultures at varying culture time			Migration indices after varying time of culture		
	0.02	0.2	4 hr	6 hr	20 hr	4 hr	6 hr	20 hr
Migration on glass*								
1	0	0	14.5	21.3	40.9	0.94	1.02	1.05
2	0	0	16.1		81.3	0.90		0.94
3	0	0	22.4		88.5	0.86		0.94
4	0	0	10.4	22.9	48.5	1.06	0.90	0.91
5	0	0	6.7	12.6	29.4	0.93	0.85	0.87
6	0	12	8.0	18.5	55.9	0.78	0.94	1.00
7	0	16	15.2	15.2	39.5	0.83	1.05	1.00
8	6		9.1	17.7	47.0	0.65	0.74	0.96
9	7		8.7	12.2	35.8	0.98	0.89	1.06
10	8		9.3	18.0	39.8	0.73	0.91	0.96
11	8		10.1	17.7	45.0	0.62	0.55	0.84
12	10		12.7	20.3	43.5	0.81	0.85	1.05
13	10		19.7	32.5	58.0	0.71	0.76	0.93
14	12		11.8	21.2	36.9	0.76	0.69	0.99
15	14		11.6	17.6	48.3	0.64	0.61	1.03
16	15		13.0	20.5	47.4	0.97	1.05	1.07
17	15		23.6	40.4	73.8	0.58	0.59	0.84
18	16		9.7	15.2	50.7	0.70	0.78	1.10
19	21		25.6	41.6	69.5	0.68	0.72	0.99
20	21		19.0	24.6	59.2	0.88	0.95	0.91
Migration on polystyrene*								
4	0	0	19.7	26.8	50.5	0.90	0.99	0.92
7	0	16	30.0	45.7	103.3	0.73	0.68	0.68
9	7		19.9	26.1	84.9	0.56	0.61	0.66
16	15		23.4	41.6	122.1	0.69	0.71	0.78
20	21		33.6	44.9	141.6	0.58	0.68	0.68

* In experiments 4, 7, 9, 16, and 20 the leukocyte migration was examined both on glass and on polystyrene.

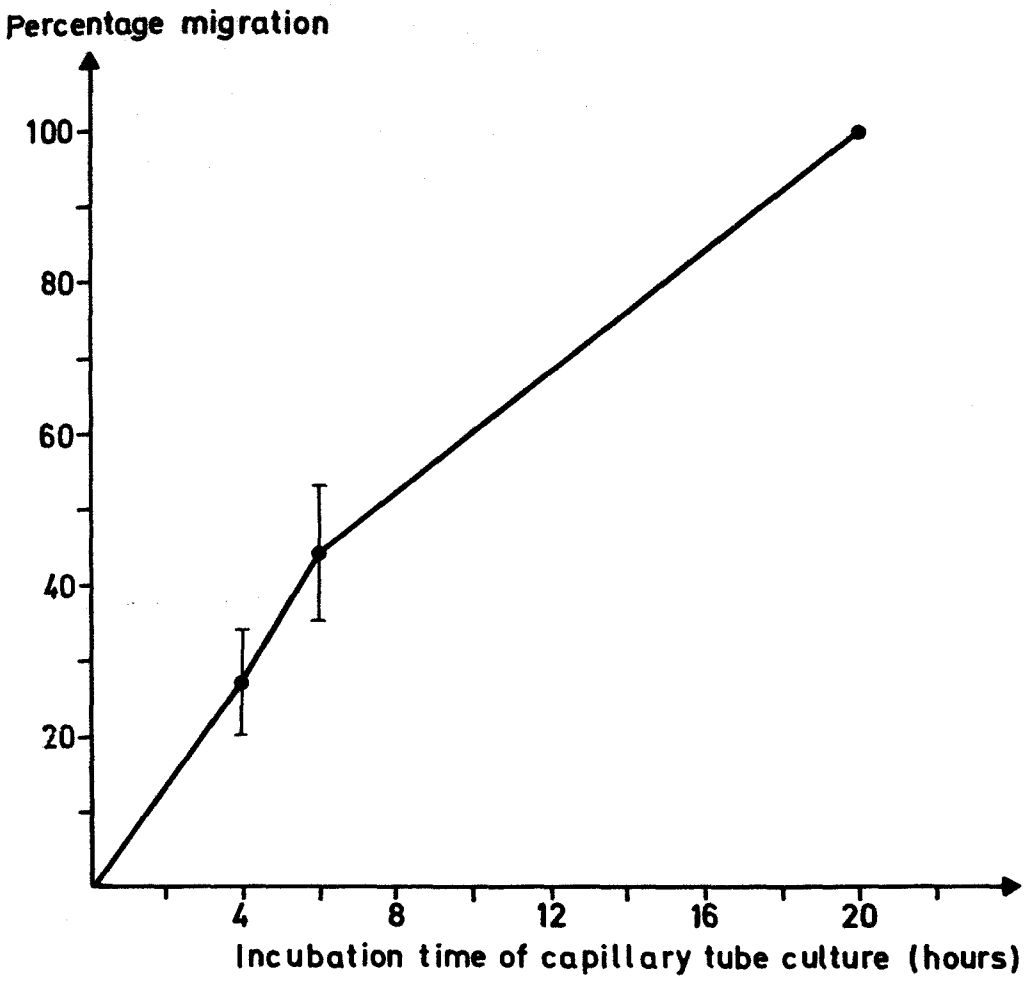

Fig. 1.

Time course of leukocyte migration using capillary tube migration technique. The leukocytes were not stimulated. Migration areas after 4 and 6 hr are expressed as per cent of the 20-hr area. Vertical lines with bars indicate ± 1 SD.

few cell were seen even close to the wall of the culture chamber. Therefore, the 20-hr measurement was omitted in following experiments.

Table 2 shows that the migration of PBL stimulated by adding PPD to the TC medium in the culture chamber (series A stimulation procedure) was inhibited a little more than that of the same leukocytes stimulated by preincubating the cells with PPD before culturing in medium without PPD (series B stimulation procedure). In average the 4-hr MI determined

by these two stimulation procedures differed only 0.04, but the difference was significant ($p < 0.02$).

TABLE 2

PPD-Induced Migration Inhibition of Capillary Tube Leukocyte Cultures. Leukocytes Were Stimulated Either by Adding PPD to the Medium of the Culture Chamber (Procedure A) or by Preincubating the Cells With PPD and Culturing in PPD-Free Medium (Procedure B). Bottoms of Culture Chambers Were Made of Polystyrene.

Exp. No.	Cutaneous reaction in mm after PPD (μg)		Procedure of stimulation	4-hr control migration area (mm^2)	4-hr migration index
	0.02	0.2			
1	0	7	A	26.4	0.80
			B	24.7	0.81
2	9		A	32.1	0.61
			B	25.6	0.71
3	10		A	27.6	0.63
			B	24.8	0.69
4	12		A	23.4	0.76
			B	20.5	0.75

Comparison Between Capillary Tube and Agarose Migration Technique

Direct PPD-induced migration inhibition of PBL. Table 3 shows a comparison between direct PPD-induced migration inhibition measured both by LMCT (4-hr MI) and by LMAT (20-hr MI). In the Mantoux-positive group, the average MI of LMCT was 0.22 higher than that of LMAT. The difference was significant ($p < 0.001$, t-test for paired comparisons).

Migration inhibition of PBL by MIF-containing supernatants. Table 4 shows the migration inhibitory effect of cell-free culture supernatants tested both by capillary tube and by agarose migration technique. The mean 4-hr MI determined by capillary tube migration technique was 0.18 higher than the mean 20-hr MI determined by agarose migration technique.

The difference was significant ($p < 0.001$, t-test for paired comparisons). In these experiments the escape from early MIF-induced migration inhibition of capillary tube cultures was as pronounced as that of direct LMCT.

TABLE 3

PPD-Induced Migration Inhibition of Human Peripheral Blood Leukocytes Demonstrated by Both Capillary Tube and Agarose Technique.

Exp. No.	Cutaneous reaction in mm after PPD (μg)		Leukocyte migration capillary tube test by procedure B		Leukocyte migration agarose test	
	0.02	0.2	4-hr control migration area (mm^2)	4-hr migration index	20-hr control migration area (mm^2)	20-hr migration index
1	0	0	31.3	0.95	39.6	0.91
2	0	10	29.6	0.81	64.3	0.68
3	0	11	18.5	0.78	34.4	0.71
4	6		19.5	0.83	53.4	0.51
5	6		12.8	0.63	37.9	0.30
6	7		24.0	0.82	28.2	0.70
7	9		21.0	0.92	35.8	0.45
8	9		31.4	0.67	27.4	0.39
9	12		22.0	0.95	63.3	0.95
10	18		33.4	0.82	52.7	0.68
11	20		15.8	0.75	47.3	0.47
12	20		18.0	0.60	38.4	0.30

DISCUSSION

Escape of leukocytes from early PPD-induced migration inhibition in the present capillary tube migration experiments was very pronounced. Using culture chambers with bottoms of glass, 10 out of 15 Mantoux-positive persons had 4-hr MI below 0.80 which was the lower limit of the normal range (95 per cent confidence limits) of the Mantoux-negative group; but after 20 hr of culture the mean MI of the Mantoux-positive persons was 0.23 higher than that after 4 hr and

TABLE 4

Migration Inhibition Factor (MIF) Activity of Cell-Free Supernatants From Mixed Lymphocyte Cultures (Sup-MLC) or PPD-Stimulated Cultures of Mononuclear Leukocytes (Sup-PPD) Determined by Both Capillary Tube and Agarose Migration Technique.

Exp. No.	MIF activity of supernatant determined by			
	capillary tube technique		agarose technique	
	4-hr control migration area mm^2	4-hr migration index	20-hr control migration area mm^2	20-hr migration index
Sup-MLC				
1	17.3	0.79	38.6	0.56
2	18.8	0.78	28.8	0.54
3	15.3	0.69	32.4	0.44
Sup-PPD type I				
4	15.0	0.72	47.7	0.67
5	16.7	0.83	36.0	0.66
6	13.8	0.97	27.8	0.63
7	14.9	0.65	25.3	0.54
8	24.4	0.56	49.7	0.49
9	14.0	0.57	40.3	0.46
10	16.2	0.50	23.6	0.44
Sup-PPD type II				
11	21.4	0.87	36.1	0.77
12	15.4	0.82	42.5	0.70
13	27.7	0.99	42.4	0.67
14	16.8	0.82	42.0	0.65
15	26.5	0.77	30.6	0.59
16	12.0	0.73	37.0	0.44
17	22.9	0.58	39.5	0.28

in none of the cases a PPD-induced migration inhibition was observed.

The escape phenomenon has also been observed in a previous study of LMAT (5). In that study, the mean 20-hr MI was only 0.08 higher than the mean 4-hr MI, and only 4 out of 40 Mantoux-positive persons had 20-hr MI above the lower limit of the normal range of Mantoux-negative persons.

The much more pronounced escape in LMCT may be caused by the lower ratio between the 4-hr and the 20-hr migration areas of unstimulated PBL in that test. In the present study the mean 4-hr migration area of LMCT was 27 (SD 7) per cent of the 20-hr area, whereas the corresponding percentage in the previous study of LMAT was 78 (SD 15) per cent. It has been proposed (5) that escape from early migration inhibition occurs, because MIF-sensitivity of the migrating polymorphonuclear leukocytes decreases during the culture period. A decreasing MIF-sensitivity in the later part of the culture period will influence the 20-hr MI of LMAT only slightly, because most of the migration takes place during the first 4 hr of culture in agarose medium. But using LMCT, in which most of the migration occurs during the 4 to 20 hr period, the effect of a decreasing MIF-sensitivity will influence the 20-hr measurement to a considerable degree. Another disadvantage by the 20-hr measurement of LMCT, in the present study, was the blurred peripheral borders of the very large migration areas at that time.

The difficulty or inability of several groups (2, 11, 12, 13, 15, 17) to demonstrate PPD-induced migration inhibition of human PBL by capillary tube migration technique may, among other things, be caused by the escape phenomenon; but unfortunately the migration inhibition in these studies were measured only after 18 to 24 hr.

In animal studies, escape of peritoneal exudate cells from PPD-induced migration inhibition has also been observed (7, 10). Corresponding to this, Marsman et al. (13) has observed that mixtures of human peripheral blood lymphocytes and guinea pig peritoneal exudate cells escape an initial PPD-induced migration inhibition during the 6 to 18 hr culture period. As no migration areas were stated in these studies it cannot be seen if most of the migration took place during the later culture period, in which the escape phenomenon occurred.

In a study of Rosenberg & David (15), PPD-induced migration inhibition after 24 hr of culture could be demonstrated only if the dense inner zones of the migration areas were meas-

ured, whereas the whole migration areas of control and PPD-stimulated cultures in many cases were the same. Measurements after a shorter culture period were not stated, but it is possible that the dense inner zones represented migration areas after a shorter culture period, at a time before escape of polymorphonuclear leukocytes occurred.

The present comparison between direct PPD-induced migration inhibition demonstrated by LMCT and that demonstrated by LMAT shows that the agarose migration technique was the most sensitive judged by lower MI and fewer false negative results. MI of LMCT were measured after 4 hr, because no PPD-induced migration inhibition could be demonstrated after 20 hr. MI of LMAT were measured after 20 hr, but this is not in advantage of LMAT, since it has been shown (5) that the 4-hr MI of LMAT is significantly lower than the 20-hr MI.

The present study shows also that agarose migration technique was much more sensitive than capillary tube migration technique estimated by the ability to demonstrate MIF-activity of cell-free culture supernatants using PBL as migrating indicator cells.

SUMMARY

Agarose migration technique was more sensitive than capillary tube migration technique both in demonstrating direct PPD-induced migration inhibition of human peripheral blood leukocytes and in demonstrating migration inhibition factor (MIF) activity of cell-free supernatants from PPD-stimulated cultures of human mononuclear leukocytes or mixed human lymphocyte cultures.

The escape of leukocytes from early PPD-induced migration inhibition was very pronounced using capillary tube migration technique. After 4 hr of culture, 10 out of 15 capillary tube leukocyte cultures from Mantoux-positive persons showed migration inhibition, but after further culture for 16 hr no inhibition could be demonstrated. This may be because about

three quarters of the migration occurred during the 4 to 20 hr culture period in which the MIF-sensitivity of polymorphonuclear leukocytes presumably decreased. The escape phenomenon was less pronounced using agarose migration technique, probably because about three quarters of the migration occurrs during the first 4 hr of agarose leukocyte cultures.

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