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LEUKOCYTE MIGRATION AGAROSE TECHNIQUE: SOME TECHNICAL DETAILS

By

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Antigen-induced inhibition of cell migration has been used as an *in vitro* correlate of cell-mediated immunity since the original study of Rich & Lewis (13). They demonstrated tuberculin-induced cell migration inhibition in cultures of spleen and buffy coat explants from guinea pigs exhibiting delayed-type hypersensitivity to tuberculin. The explant migration technique has later on been elaborated especially by Švejcar et al. (14).

The capillary tube migration technique described by George & Vaughan (11) and further elaborated in guinea pig experiments by David et al. (9) was a considerable technical advance since it was easier to standardize and more reproducible than the explant technique. In 1967, Søborg & Bendixen (15) showed that the capillary tube technique could be used also to demonstrate cell-mediated immunity in man using peripheral blood leukocytes (PBL) as migratory cells.

In 1968, Carpenter et al. (2) described an agar technique for demonstration of antigen-induced inhibition of guinea pig cell migration. In the following years, Clausen (3) developed an agarose migration technique suitable for the study of tuberculin immunity in man. The agarose technique has been used both for demonstration of direct antigen-induced PBL migration inhibition (3, 6, 7, 8) and for demonstration of migration inhibitory activity of cell-free supernatants from

cultures of PPD-stimulated mononuclear leukocytes from Mantoux-positive persons (5) and from mixed human lymphocyte cultures (4). Even if the results of the direct leukocyte migration agarose test (LMAT) have been confirmed by others (1, 10, 12), I have, by personal communications, learned that other investigators have met difficulties when attempting to use the agarose technique.

In the present study some technical details of the agarose migration technique will be presented.

MATERIALS AND METHODS

1) *Tissue Culture Media*

Liquid Medium

Hepes-buffered TC medium was prepared from 100 ml ten-fold concentrated TC 199 (TC-medium 199, 10 X, Difco Laboratories, Detroit, Mich., U.S.A.), 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.

Agarose Medium

Standard Procedure

The final gel medium was composed of 1 per cent agarose (Litex, Glostrup, Denmark), 66 i.u./ml penicillin and 66 μ g/ml streptomycin (TC penicillin-streptomycin, desiccated, Difco Laboratories), 10 per cent horse serum (Statens Seruminstitut, Copenhagen, Denmark), and 10 per cent ten-fold concentrated TC 199 in distilled water. The constituent parts were mixed at 47° C. 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 were poured into disposable 50-mm plastic Petri dishes (Millipore Filter Corp., Bedford, Mass., U.S.A.). After the medium had solidified, six to eight wells of 2.3 mm diameter were punched out in the gel.

When leukocytes had been placed in the wells, the agarose plates were incubated at 37° C in a humidified atmosphere of 2 per cent CO₂ in air.

Deviation from Standard Procedure

Agarose concentration. In 8 experiments, the migration of non-stimulated PBL in agarose medium with 1 per cent agarose was compared with that in media with 0.75, 1.25, and 1.50 per cent agarose.

Hepes-buffered agarose medium. In 22 experiments, migration inhibition in standard medium was compared with that in a Hepes-buffered agarose medium. This medium was composed of 1 per cent agarose, 66 i.u./ml penicillin, 66 $\mu\text{g}/\text{ml}$ streptomycin, 10 per cent horse serum, 0.62 g/100 ml Hepes, and 10 per cent ten-fold concentrated TC 199 in distilled water. The pH of the medium was adjusted to 7.30 with 5 N NaOH.

When the leukocytes had been placed in wells in the medium, the agarose plates were incubated at 37° C in humidified atmospheric air.

2) Direct PPD-Induced Migration Inhibition

Standard Procedure

Venous blood was collected in 10-ml polysterol tubes (Nuncion tubes, Nunc, Roskilde, Denmark) each containing 250 i.u. heparin (Heparin LEO, 5000 i.u. per ml, Leo, Copenhagen, Denmark) and 2 ml 5 per cent dextran 250 (Dextran 250, m.w. 250,000, Pharmacia, Uppsala, Sweden) in saline (0.9 per cent) solution. After sedimentation at 37° C for 1 hr, the leukocyte-rich plasma was withdrawn, centrifuged at 220 x g for 5 min, and the cells washed three times in Hanks' balanced salt solution. The cells were then resuspended in Hepes-buffered TC 199 with 10 per cent horse serum.

Purified protein derivative of tuberculin (PPD) in phosphate buffer (Statens Seruminstitut) 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 $2.1 \times 10^8/\text{ml}$. After incubation at 37° C for $\frac{1}{2}$ hr, six to eight 7- μl portions from each PBL suspension were placed in wells in agarose medium. After incubation at 37° C for 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.

Deviation from Standard Procedure

Cell concentration. In 13 experiments, direct PPD-induced migration inhibition using the standard cell concentration of $2.1 \times 10^8/\text{ml}$ (i.e. 1.5×10^6 per 7 μl , which was the volume of leukocyte suspension placed in the wells) was compared with that obtained with lower (0.38 and $0.75 \times 10^6/7 \mu\text{l}$) or higher ($3.00 \times 10^6/7 \mu\text{l}$) cell concentrations.

Lysis of contaminating erythrocytes. In 7 experiments, PPD-induced migration inhibition obtained by the standard procedure was compared with that obtained when the leukocyte suspension had been incubated with 0.83 per cent NH_4Cl in the ratio of 1 part cell suspension to 8 parts NH_4Cl for 10 min.

PPD-preincubation time. In 45 experiments, migration inhibition of PBL after preincubation with PPD for $\frac{1}{2}$ hr was compared with that obtained when the

cells were placed in wells in agarose medium just after addition of PPD (11 experiments) or after preincubation with PPD for 1½ hr (34 experiments).

3) Migration Inhibition by Culture Supernatants

MIF-containing supernatants from mixed lymphocyte cultures or from PPD-stimulated cultures of mononuclear leukocytes from Mantoux-positive persons were prepared as previously described (4, 5).

Standard Procedure

PBL prepared as described above or purified polymorphonuclear leukocytes (PMN) prepared as previously described (4) were divided into 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 PBL/ml or 1.4×10^8 PMN/ml. After incubation at 37° C for 1½ hr, six 7-μl portions from each cell suspension were placed in wells in agarose medium. Migration areas were measured after 20 hr. The migration inhibitory effect of a supernatant from a stimulated culture was expressed as a MI which indicated the ratio between the average migration area of indicator cells resuspended in supernatant from the stimulated cultures and that of cells resuspended in supernatant from the corresponding control cultures.

In 6 experiments, the migration inhibition of PBL in agarose medium buffered with CO₂/bicarbonate was compared with that in agarose medium buffered with Hepes.

Deviation from Standard Procedure

PMN concentration. In 4 experiments, migration inhibition by culture supernatants from mixed lymphocyte cultures using the standard cell concentration of 1.4×10^8 PMN per ml (i.e. 1.0×10^6 per 7 μl, which was the volume placed in the wells) was compared with that obtained using lower (0.50 and 0.75×10^6 /7 μl) or higher (1.50×10^6 /7 μl) PMN concentrations.

RESULTS

Role of Agarose Concentration (Table 1)

The leukocyte migration areas using 0.75 per cent agarose medium were about the same as those obtained using the standard 1.00 per cent agarose medium. In agarose media with 1.25 and 1.50 per cent agarose, the cells migrated less than in the standard medium. The peripheral borders of migration zones in medium with 0.75 per cent agarose were a

TABLE I

Role of Agarose Concentration on Migration of Non-Stimulated Human Peripheral Blood Leukocytes.

Exp. No.	Migration areas (mm ²) in agarose media of varying agarose concentration (per cent)			
	0.75	1.00	1.25	1.50
1	54.6	59.6		
2	54.4	51.7	46.1	34.5
3	43.8	41.5	33.5	
4	33.1	39.4		
5	35.5	36.6	26.6	21.6
6	31.6	27.6	24.9	
7	19.0	19.0	16.5	
8	16.8	11.2	9.3	

little blurred making the measurement difficult; but at higher agarose concentrations the borders were always well-defined.

Migration Inhibition in Hepes-Buffered Agarose Medium Compared with that in CO₂/Bicarbonate-Buffered Medium (Table II)

Direct PPD-induced migration inhibition. The control migration areas in Hepes-buffered medium were smaller than those in CO₂/bicarbonate-buffered medium, the mean migration areas being 33.3 and 45.0 mm², respectively. This difference was significant ($p < 0.001$, t-test for paired comparisons). The peripheral borders of the migration zones were well-defined in both media. PPD-induced migration inhibition was most pronounced in CO₂/bicarbonate-buffered medium. However, the mean difference between MI in the two media was only 0.05, but the difference was significant ($0.025 < p < 0.05$, t-test for paired comparisons).

Migration inhibition of PBL by culture supernatants. Neither migration areas, nor migration inhibitions were significantly different in the two media ($p > 0.1$, t-test for paired comparisons).

TABLE 2

Leukocyte Migration Inhibition in Hepes-Buffered Agarose Medium Compared with that in CO₂/Bicarbonate-Buffered Medium.

Exp. No.	Agarose medium buffered with			
	Hepes		CO ₂ /bicarbonate	
	migration area (mm ²) of control culture	migration index	migration area (mm ²) of control culture	migration index
Direct PPD-induced migration inhibition				
1	32.1	0.89	58.8	0.84
2	26.8	0.95	47.2	0.82
3	52.6	0.79	51.5	0.75
4	21.5	0.74	33.4	0.70
5	31.4	0.62	37.6	0.65
6	47.7	0.67	63.8	0.62
7	31.2	0.58	34.4	0.61
8	17.8	0.56	21.3	0.54
9	9.3	0.48	9.9	0.53
10	38.5	0.44	51.2	0.42
11	49.5	0.54	62.8	0.41
12	43.4	0.49	59.1	0.40
13	31.4	0.41	39.9	0.40
14	28.8	0.42	33.5	0.38
15	19.9	0.59	41.9	0.35
16	50.3	0.37	73.5	0.31
Migration inhibition by cell-free supernatants from PPD-stimulated cultures of mononuclear leukocytes				
17	22.0	0.82	34.7	0.81
18	19.7	0.72	27.7	0.74
19	17.5	0.71	22.4	0.66
20	19.8	0.54	20.2	0.58
21	49.5	0.45	49.7	0.52
22	32.6	0.42	27.7	0.49

Role of Cell Concentration

Direct PPD-induced migration inhibition (Table 3). Control migration areas increased with the cell concentration. At 0.38×10^6 cells/7 μ l no migration was seen from four of the cultures; and in the other experiments using this cell concen-

TABLE 3

Role of Cell Concentration on Leukocyte Migration and Direct PPD-Induced Migration Inhibition.

Exp. No.	Number of leukocytes per culture (millions per 7 μ l)							
	0.38		0.75		1.50		3.00	
	migration area (mm ²) of control culture	MI	migration area (mm ²) of control culture	MI	migration area (mm ²) of control culture	MI	migration area (mm ²) of control culture	MI
1	0*		14.6	0.84	24.3	0.89	36.9	0.95
2	10.8	0.79	23.1	0.76	40.6	0.80	46.9	0.82
3					38.8	0.72	45.8	0.76
4					53.5	0.69	58.3	0.84
5					53.6	0.66	70.1	0.58
6	15.4	0.55	30.2	0.56	41.9	0.65	52.1	0.70
7	5.4§	0.81	9.7§	0.99	13.5	0.64	62.7	0.88
8	0		5.4§	0.60	9.7	0.61	15.8	0.76
9	0		16.7	0.60	27.7	0.58	42.6	0.63
10			8.8§	0.55	15.9	0.50	25.5	0.47
11	7.8	0.46	18.2	0.43	46.0	0.44	54.2	0.62
12	0		8.8	0.42	19.3	0.38	30.3	0.43
13					46.2	0.33	51.1	0.71

* 0 indicates that there was no significant migration.

§ The peripheral border of the migration area was so blurred that the measurement of the area must be considered uncertain.

tration, the peripheral borders of the migration zones were ill-defined making the measurement unreliable. Even at 0.75×10^6 cells/7 μ l, three of the cultures had ill-defined migration borders. The other cultures with 0.75×10^6 cells/7 μ l had well-defined borders, and their MI did not differ significantly from those obtained with cultures of 1.50×10^6 cells/7 μ l ($p > 0.1$, t-test for paired comparisons). The migration inhibition of 3.00×10^6 /7 μ l cultures was less than that of 1.50×10^6 /7 μ l cultures. The mean MI difference was 0.10, which was significant ($p < 0.02$).

Migration inhibition of purified polymorphs by culture supernatants (Table 4). Control migration areas increased

TABLE 4

Role of Concentration of Polymorphonuclear Leukocytes Used as Migrating Indicator Cells in the Study of Migration Inhibitory Effect of Cell-Free Supernatants from Mixed Lymphocyte Cultures.

Exp. No.	Number of polymorphonuclear leukocytes per culture (millions per 7 μ l supernatant)							
	0.50		0.75		1.00		1.50	
	migration area (mm ²) of control culture	MI	migration area (mm ²) of control culture	MI	migration area (mm ²) of control culture	MI	migration area (mm ²) of control culture	MI
1	36.1	0.34	46.3	0.42	50.8	0.49	56.0	0.49
2	6.3	0.54	11.6	0.47	18.5	0.48	21.5	0.47
3	26.6	0.36	36.7	0.36	38.6	0.44	42.5	0.44
4	16.2	0.33	22.2	0.32	21.7	0.44	27.9	0.42

with the PMN concentration. However, in these experiments, the migration inhibition was independent of the cell concentration. The peripheral borders of migration zones were all well-defined, even at a PMN concentration of $0.50 \times 10^6/7 \mu\text{l}$.

Effect of NH₄Cl Erythrocyte Lysis (Table 5)

The migration areas from control cultures in which the erythrocytes had been lysed by NH₄Cl were smaller than those from non-lysed cultures. The mean difference was 5.6 mm², which was significant ($p < 0.025$, t-test for paired comparisons). The MI of lysed cultures were the same as those of corresponding non-lysed cultures ($p > 0.1$, t-test for paired comparisons).

Role of the PPD-Preincubation Time (Table 6)

The mean control migration area of leukocytes placed immediately in agarose medium was 38.2 mm² and that of the same leukocytes preincubated for $\frac{1}{2}$ hr was 37.4 mm². This difference was not significant ($p > 0.1$, t-test for paired comparisons). The mean control migration area of leukocytes

TABLE 5

Effect of NH_4Cl Lysis of Erythrocytes Contaminating the Leukocyte Preparation on Migration and PPD-Induced Migration Inhibition.

Exp. No.	Erythrocytes present		Erythrocytes lysed	
	migration area (mm^2) of control culture	migration index	migration area (mm^2) of control culture	migration index
1	40.4	0.75	34.4	0.73
2	34.1	0.73	20.4	0.72
3	32.0	0.70	24.7	0.62
4	25.7	0.67	22.7	0.63
5	23.1	0.58	17.7	0.65
6	20.9	0.42	22.9	0.41
7	25.9	0.33	20.4	0.54

preincubated for $1\frac{1}{2}$ hr was 30.8 mm^2 and that of the same leukocytes preincubated for only $\frac{1}{2}$ hr was 34.8 mm^2 . This difference was significant ($p < 0.001$, t-test for paired comparisons). The direct PPD-induced migration inhibition of leukocytes placed in agarose medium just after addition of PPD was less than that of the same leukocytes preincubated with PPD for $\frac{1}{2}$ hr. On average the MI difference was 0.14, which was significant ($p < 0.001$, t-test for paired comparisons). The MI of leukocytes preincubated for $1\frac{1}{2}$ hr was the same as that of leukocytes preincubated for $\frac{1}{2}$ hr.

DISCUSSION

The present study shows that the agarose concentration of the gel medium as well as the cell concentration of the leukocyte suspension placed in agarose medium determine whether the peripheral borders of the migration zones will be well-defined. Ill-defined borders were seen in agarose medium with 0.75 per cent agarose, but not in media with higher agarose concentrations. At a cell concentration of 0.38×10^6 per $7 \mu\text{l}$ (which was the volume of leukocyte suspension placed in each well in

TABLE 6

Leukocyte Migration Inhibition After Varying Time of Preincubation with PPD.

Exp. No.	Leukocytes placed in wells in agarose medium					
	just after addition of PPD		after incubation with PPD for			
	migration area (mm ²) of control culture	migration index	migration area (mm ²) of control culture	migration index	migration area (mm ²) of control culture	migration index
1	61.9	0.99	57.5	0.99		
2	43.0	0.96	38.2	0.92		
3	42.2	0.94	38.1	0.89		
4	29.6	0.88	29.4	0.72		
5	24.7	0.89	34.2	0.66		
6	48.9	0.66	40.6	0.62		
7	31.5	0.73	27.2	0.58		
8	36.6	0.51	43.3	0.36		
9	29.3	0.39	30.3	0.31		
10	45.0	0.36	49.6	0.29		
11	27.2	0.49	23.5	0.27		
12			38.8	0.95	36.4	0.94
13			39.0	0.92	40.5	0.96
14			64.0	0.89	58.6	0.90
15			33.7	0.78	23.5	0.84
16			55.8	0.73	42.8	0.82
17			27.9	0.70	22.1	0.77
18			31.8	0.68	28.6	0.52
19			39.7	0.67	31.7	0.74
20			29.0	0.67	37.1	0.72
21			34.7	0.67	34.9	0.76
22			32.1	0.66	28.6	0.61
23			38.3	0.66	33.4	0.61
24			26.5	0.66	23.9	0.68
25			39.5	0.66	31.7	0.75
26			37.5	0.65	31.1	0.67
27			19.8	0.58	16.9	0.66
28			23.6	0.54	17.8	0.45
29			30.1	0.49	36.4	0.48
30			36.2	0.49	32.1	0.49
31			27.9	0.48	25.1	0.44
32			28.7	0.47	28.5	0.37
33			16.1	0.46	14.3	0.43
34			40.0	0.45	29.3	0.39

TABLE 6 (continued)

Exp. No.	Leukocytes placed in wells in agarose medium					
	just after addition		after incubation with PPD for			
	of PPD		1/2 hr		1 1/2 hr	
	migration area (mm ²) of control culture	migration index	migration area (mm ²) of control culture	migration index	migration area (mm ²) of control culture	migration index
35			36.3	0.44	31.9	0.46
36			36.6	0.41	34.9	0.39
37			29.9	0.38	22.9	0.34
38			33.0	0.36	24.9	0.31
39			27.8	0.35	24.4	0.35
40			41.8	0.35	35.8	0.37
41			29.7	0.33	26.9	0.33
42			40.1	0.30	35.0	0.30
43			46.6	0.28	42.2	0.29
44			36.6	0.24	38.3	0.27
45			34.1	0.22	26.3	0.22

the gel medium) migration zones had ill-defined borders, a phenomenon also seen in some cultures with a cell concentration of $0.75 \times 10^6/7 \mu\text{l}$; but never if the cultures contained 1.00×10^6 cells or more per $7 \mu\text{l}$. At 3.00×10^6 cells per $7 \mu\text{l}$, the PPD-induced migration inhibition was less than that obtained with 1.00 or $1.50 \times 10^6/7 \mu\text{l}$, probably caused by less favorable culture conditions at the high cell concentration. The indirect MIF assays demonstrate that when purified polymorphs are used as migrating indicator cells, well-defined peripheral borders can be obtained with cell concentrations as low as 0.50×10^6 per $7 \mu\text{l}$.

The present experiments show that the CO_2 /bicarbonate-buffered agarose medium can be substituted by a Hepes-buffered medium, which requires simpler incubation conditions since the gassing with CO_2 is avoided. The smaller migration areas in Hepes-buffered medium may explain the slightly less PPD-induced migration inhibition in this medium.

In capillary tube leukocyte cultures, a high erythrocyte con-

tamination of the leukocyte preparation results in greater migration areas and less antigen-induced migration inhibition; but in LMAT using leukocyte preparations with an erythrocyte number between 30 and 80 per cent of the leukocyte number, addition of purified erythrocytes increasing the number to 2-3 times that of leukocytes does not influence neither the area of migration zones nor the PPD-induced migration inhibition (Clausen, published experiments). This difference between the capillary tube technique and the agarose technique is probably caused by the fact that erythrocytes slide out of the capillary tube and contaminate most of the migration zone, but do not leave the well in agarose medium (3). In accordance with the independence of LMAT to erythrocyte contamination, the present experiments demonstrate that lysis of the contaminating red cells does not alter PPD-induced migration inhibition in agarose medium. The migration areas of lysed cultures in the present study were smaller than those of the corresponding non-lysed cultures. This may be because the lysis procedure damages the leukocytes, although it does not affect the migration inhibition reaction. Thus, erythrocyte lysis of the cell suspension is unnecessary with respect to migration inhibition and should be avoided.

The presented data show that a PPD preincubation time of $\frac{1}{2}$ hr or $1\frac{1}{2}$ hr induces the same migration inhibition; but if the cells are placed in wells in agarose medium just after addition of PPD, the inhibition is less pronounced. Corresponding to this, it has been shown in a previous study (3) that no or only a slight migration inhibition was induced when leukocytes without PPD-preincubation were cultured in PPD-containing agarose medium, even if a pronounced migration inhibition was induced when the same cells were preincubated with PPD for $\frac{1}{2}$ hr. Thus, preincubation with PPD is decisive for obtaining a high degree of PPD-induced migration inhibition.

The smaller migration areas of cells preincubated for $1\frac{1}{2}$ hr compared with those of cells preincubated for only $\frac{1}{2}$ hr may be caused by the unfavourable culture conditions during the longer preincubation period.

SUMMARY

Some technical details concerning the agarose migration technique used for demonstration of tuberculin immunity in man were reported. An agarose concentration of 1 per cent was suitable; at lower concentrations the peripheral border of the migration zones was ill-defined, at higher concentrations the migration areas became smaller. Hepes-buffered gel medium could be used instead of CO₂/bicarbonate-buffered medium.

In the direct leukocyte migration agarose test (LMAT), a leukocyte number of $1.0-1.5 \times 10^6$ per 7 μ l, which was the culture volume placed in wells in agarose medium, was to prefer. At lower cell concentrations the migration areas were small and the peripheral borders ill-defined. At higher concentration the migration inhibition was less pronounced. Hemolysis of erythrocytes contaminating the leukocyte preparation was unnecessary and probably damaging to the leukocytes, since it resulted in smaller migration areas, even if the migration inhibition was unaltered. Leukocytes preincubated with PPD for $\frac{1}{2}$ hr before being placed in wells in agarose medium were inhibited as much as leukocytes preincubated for $1\frac{1}{2}$ hr, but cells placed in agarose medium just after addition of PPD were inhibited considerably less.

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