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Leucocyte motility in the newborn: determination of spontaneous movement is essential in the *in vitro* assessment of neutrophil chemotaxis

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SUMMARY

Polymorphonuclear leucocytes (PMNs) of the newborn show poor movement *in vitro* toward a chemotactic stimulus, such as zymosan-activated human serum (ZAS). This may result from a defect either in spontaneous movement or in the response of the cells to the stimulus. To identify the defect we studied chemotaxis, chemokinesis and spontaneous movement of cord blood PMNs by agarose assay and by the leading front modification of the Boyden chamber technique. Under agarose, cord PMNs showed much spontaneous movement. This, associated with poor stimulated movement, indicated a defect in the responsiveness of cord PMNs to ZAS. In the membrane filter, cord PMNs migrated spontaneously, and in the presence of ZAS, significantly less than adult cells. The weak spontaneous movement most probably resulted from poor deformability of cord PMNs and contributed to the weak stimulated movement of these cells in the filter. Our results indicate that the impaired chemotaxis was due to a defect in both responsiveness and deformability of cord PMNs rather than to a defect in their intrinsic ability to move.

INTRODUCTION

Movement of polymorphonuclear leucocytes (PMNs) toward an attractant in *in vitro* tests, e.g. the leading front modification of Boyden's method (Zigmond & Hirsch, 1973) or migration under agarose (Nelson, Quie & Simmons, 1975; John & Sieber, 1976; Repo, 1977), is the resultant of spontaneous movement, the chemokinetic response, and orientation (terms defined by Keller *et al.*, 1977). A defect in any of these three features can result in decreased cell attraction.

Blood PMNs from newborn babies or umbilical cords show poor movement *in vitro* toward a chemotactic stimulus in comparison with that of adult PMNs (Miller, 1971; Klein *et al.*, 1976; Pahwa *et al.*, 1977). Furthermore, Miller (1975) showed that the membrane of neonate PMNs is much more rigid than that of adult cells, thereby reducing deformability. Using the agarose assay and the leading front method in parallel, we examined the chemotactic defect in cord PMNs to see whether it is due to impaired spontaneous movement or to impaired responsiveness to an attractant.

MATERIALS AND METHODS

Blood samples. Normal full-term deliveries at the Department of Obstetrics and Gynaecology,

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University Central Hospital, Helsinki, were selected. Twenty to 40 ml of heparinized (25 iu/ml, preservative-free heparin, Medica Company, Helsinki) arterial and venous blood was collected immediately after the cutting of the cord. Adult blood samples were drawn from antecubital veins of healthy volunteers aged 20 to 40 years within 10 min after the cord blood samples had been collected.

Cells. Buffy coat cells were separated by the two-phase method (Böyum, 1974) as described previously (Repo, 1977), washed three times in Hanks' balanced salt solution (HBSS), and resuspended in the cell medium at $5{\cdot}0 \times 10^7$ PMNs/ml for the agarose assay and at $1{\cdot}0 \times 10^6$ PMNs/ml for the membrane filter assay.

Attractants. Zymosan-activated serum (ZAS) was prepared as described previously (Repo, Kostiala & Kosunen, 1978). To make a reference solution for ZAS, 1·0 ml of inactivated (30 min at 56°C) pooled normal human serum was mixed with 0·1 ml of zymosan (10 mg/ml in saline, Sigma Chemical Company, St Louis, Missouri), and zymosan particles were removed by centrifugation. A 2·5% casein solution was prepared by dissolving casein (Hammarsten, E. Merck, Darmstadt) in HBSS with alkali, followed by neutralization with HCl.

Agarose assay. The determination of the chemotactic, the chemokinetic and the spontaneous movement of PMNs was performed by the agarose assay as described (Repo *et al.*, 1978; Repo *et al.*, 1979). Briefly, agarose medium was 1% agarose (Biomedical Division of Marine Colloids Incorporated, Rockland, Maine) and 1% human serum albumin (HSA) (AB Kabi, Stockholm) in medium 199 (GIBCO-BIOCULT, Glasgow) with antibiotics. Agarose medium in 5-ml volumes was applied to disposable tissue culture dishes (Falcon Plastics, Oxnard, California). Pairs of wells were then cut in the agarose gel. In each pair, the two wells, one for the attractant ZAS or its reference solution and the other for the cells, were 3 mm apart. ZAS in portions of 5 μl was applied to its well twice: 60 min before and simultaneously with the application of a 5-μl sample of the cell suspension to the cell well. After incubation for 4, 8 or 20 hr at 37°C in a mixture of 2% CO_2 in air, a 10% formaldehyde solution was applied as a fixative to the dishes for 24 hr. The distances of migration toward ZAS (chemotaxis) or toward its reference solution (spontaneous movement) were determined (Repo, 1977). To study enhanced random movement (chemokinesis), different dilutions of ZAS (the final proportions of ZAS in agarose, v/v, 0·5, 2·5, 12·5 and 32·5%) were incorporated in the agarose medium. Single wells were cut in the agarose gels and filled with the cell suspension. After incubation and fixation as above, the distance of migration was determined (Repo *et al.*, 1978). Each migration assay was performed in quadruplicate.

Membrane filter assay. The leading front modification (Zigmond & Hirsch, 1973) of the Boyden chamber technique (Boyden, 1962) was used according to Wilkinson (1974). Buffy coat cells containing $2{\cdot}0 \times 10^5$ PMNs were placed on 3-μm pore size membrane filters (Millipore Corporation). Two types of spontaneous movement assays were performed: PMNs were allowed to migrate either in HBSS or in HBSS containing 1% HSA. When the effect of the filter pore size on PMN movement was studied, 5- and 8-μm pore size membrane filters were also used, and PMN movement either in HBSS or toward 12·5% ZAS was determined. To study directional movement, a chequer-board was made by varying the concentration of casein (0, 0·5, 1, 2, 4 and 8 mg/ml in HBSS) on both sides of the filter. After incubation for 50 min as above, the filters were processed for microscopy. Distance of the cell front from the starting point was determined as an average of five microscopical fields (× 400) of each of the triplicate filters.

Statistical evaluation. Paired sample *t*-test was used in the comparison of cord and adult PMNs.

RESULTS

Spontaneous movement

In seventeen pairs of cord and adult blood samples examined by the agarose technique cord PMNs migrated significantly further than did adult cells (Fig. 1a). This implies that the intrinsic ability for movement of cord PMNs was high. In the 3-μm pore size membrane filter without HSA, spontaneous movement of cord PMNs was significantly less than that of adult cells (Fig. 1b). This poor movement was also demonstrated in tests with 5- and 8-μm pore size membrane filters (Fig. 2).

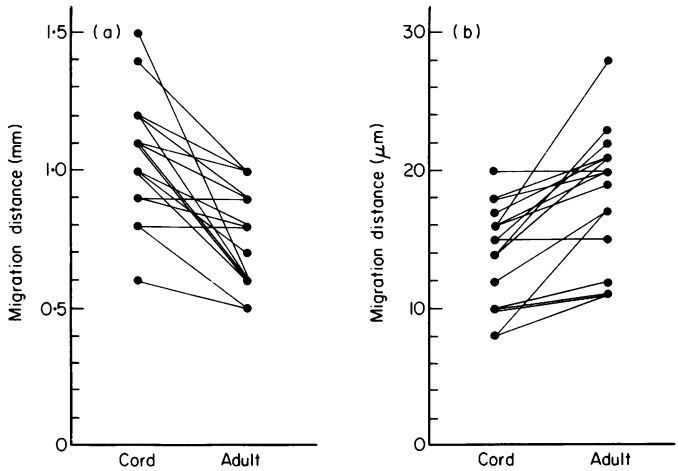

Fig. 1. Spontaneous movement of cord and adult PMNs under agarose (a) and in 3-μm pore size membrane filter without human serum albumin (b). Incubation period in agarose assay was 4 hr. The difference between cord and adult PMNs was significant ($P < 0{\cdot}001$) in both (a) and (b).

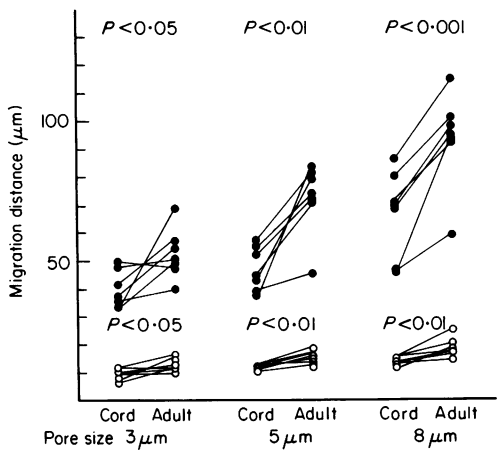

Fig. 2. Stimulated movement of cord and adult PMNs toward 12·5% zymosan-activated serum (●—●) and spontaneous locomotion without human albumin (o—o) in 3-, 5- and 8-μm pore size membrane filters. The significance of the difference between cord and adult PMNs is indicated above each group.

Because the distances of migration were short, thus resulting in very small differences between cord and adult cells, the test was repeated with HSA in the medium, which stimulates random movement of PMNs (Keller *et al.*, 1978). Nine pairs of blood samples were studied. HSA stimulated movement of both cord and adult PMNs, but the former cells were always slower than the latter (Table 1). The results indicate that, despite the high intrinsic ability for movement of cord PMNs, they migrated spontaneously less than adult cells in the membrane filter.

Stimulated movement under agarose

Six pairs of cord and adult blood samples were examined, and the migration of PMNs toward the reference solution (spontaneous movement), that toward ZAS (chemotaxis) and that under agarose mixed with ZAS (chemokinesis) were determined (Table 2). Both the concentration of ZAS and the incubation period in the assay were varied in order to demonstrate the maximum migration. Cord PMNs migrated significantly less than adult cells toward ZAS. Because the spontaneous movement of cord PMNs under agarose was not impaired, their poor migration toward ZAS

Table 1. Spontaneous movement of cord and adult PMNs in 3-μm pore size membrane filter in 1% human serum albumin

Blood sample pair	Migration distance (μm)	
	Cord*	Adult
1	66	87
2	80	87
3	71	83
4	72	115
5	80	90
6	60	75
7	60	71
8	60	64
9	52	74

* Significantly different from adult PMNs ($P < 0.01$).

Table 2. Stimulated movement of cord and adult PMNs under agarose

Direction of movement	Migration distance* (mm) after incubation for:					
	4 hr		8 hr		20 hr	
	Cord	Adult	Cord	Adult	Cord	Adult
Toward reference solution†	1·1§	0·8	1·3§	1·1	1·3§	1·1
Toward ZAS‡	1·6¶	2·4	2·0¶	2·9	2·2¶	3·0
Toward ZAS diluted 1:2	1·4	1·8	1·8§	2·3	1·8	2·3
Toward ZAS diluted 1:4	1·2	1·2	1·5	1·6	1·5	1·8
Under agarose mixed with ZAS (v/v)						
0·5%	1·0	1·0	1·2	1·2	1·4	1·1
2·5%	1·2	1·4	1·5§	1·7	1·6	1·8
12·5%	1·3	1·4	1·6	1·8	1·9	2·1
32·5%	1·1	1·2	1·4	1·4	1·6	1·6

* Mean of six blood samples.
† Mixture of heated (30 min, 50°C) human serum and zymosan.
‡ Zymosan-activated human serum.
§ Significantly different ($P < 0.05$) from adult PMNs.
¶ Significantly different ($P < 0.001$) from adult PMNs.

may have resulted from either impaired chemotactic or impaired chemokinetic response, or from both.

The chemokinetic movement of cord PMNs was consistently less than that of adult cells (Table 2). In fact, movement by the cord PMNs was slight compared with the active spontaneous movement. The results suggest that the chemokinetic responses of the newborn PMNs were impaired. To study the ability of cord PMNs to respond directionally, the leading front method was used.

Stimulated movement in membrane filter

Cord PMNs migrated significantly less than adult cells toward 12·5% ZAS in 3-, 5- and 8-μm pore

Table 3. Stimulated movement of cord PMNs in 3-μm pore size membrane filter

Above filter	Migration distance* (μm) in casein (mg/ml) Below filter					
	0	0·5	1·0	2·0	4·0	8·0
0	19					
0·5		*69*	55 (70)	68 (66)	75 (66)	77 (66)
1·0		64 (70)	*71*	58 (68)	69 (67)	73 (68)
2·0		58 (62)	62 (62)	*59*	54 (58)	64 (59)
4·0		50 (53)	50 (59)	49 (56)	*55*	63 (55)
8·0		47 (60)	52 (60)	49 (57)	55 (57)	*57*

* Migration distances to the right of figures in italic show migration in a positive gradient (chemotactic locomotion), and those to the left of italic figures indicate migration in a negative gradient. The figures in italic from the top left to the bottom right show migration in an increasing concentration of casein without a gradient (chemokinetic locomotion). Figures in parentheses, calculated according to Zigmond & Hirsch (1973), are estimates for cells which are assumed to detect the absolute concentration of casein but not the presence of a gradient.

Table 4. Stimulated movement of adult PMNs in 3-μm pore size membrane filter

Above filter	Migration (μm) in casein (mg/ml)* Below filter					
	0	0·5	1·0	2·0	4·0	8·0
0	*25*					
0·5		*75*	71 (74)	79 (76)	98 (77)	98 (70)
1·0		62 (74)	*73*	102 (75)	90 (76)	100 (71)
2·0		63 (78)	76 (77)	*79*	78 (80)	65 (63)
4·0		58 (80)	52 (79)	62 (81)	*82*	82 (75)
8·0		53 (60)	52 (59)	60 (61)	66 (62)	*55*

* See footnote to Table 3.

size membrane filters (Fig. 2). This most probably resulted from both poor spontaneous movement in the filter and their unresponsiveness to the stimulus.

To study the different components of PMN movement, a chequer-board test was made by varying the concentration of casein on both sides of the membrane filter. Three pairs of blood samples were studied, and the results obtained with one cord (Table 3) and one adult (Table 4) blood sample tested in parallel are presented as an example. The chemokinetic movement of cord PMNs (italic figures) was much less than that of adult cells. It appears that defective spontaneous movement of cord PMNs may have contributed to their poor chemokinetic movement in the filter. In the presence of a positive gradient (migration rates to the right of the italic figures), the values observed for cord PMNs were frequently higher, and in the presence of the negative gradient (to the left of the italic figures), they were consistently lower than the corresponding calculated values. This suggests that cord PMNs were capable of responding directionally. However, the migration rates of cord PMNs (Table 3) were much less than those of adult PMNs (Table 4).

DISCUSSION

Our results confirm earlier findings that PMNs from newborn babies or cord blood show less stimulated movement than those of adults when tested by the membrane filter technique (Miller, 1971; Pahwa *et al.*, 1977; Miler, Vondraček & Hromádková, 1979) or by the agarose technique (Klein *et al.*, 1976). As, in our tests, spontaneous movement of PMNs from cord blood was very active, it is believed that the poor response of the cord PMNs to the stimulus of ZAS is due to a defect in responsiveness to the stimulus rather than impaired motility. We recently showed that PMNs from patients with Pelger-Hüet anomaly had impaired spontaneous movement as well as poor stimulated movement, and thus differed from the results obtained with cord blood PMNs (Repo *et al.*, 1979).

In the study reported here, cord blood PMNs showed poor spontaneous movement in membrane filters, though apparently migrated like those of normal adults in agarose. The discrepancy may be due to the membrane of PMNs of neonates being more rigid than that of adult cells, as demonstrated by Miller (1975), and thereby less able to penetrate the pores of the filter. The present results suggest that less deformation of a cell is required for migration under agarose than in the membrane filter, as propounded by Nelson (1978). On the other hand, the membrane filter technique may be more sensitive than the agarose assay in the evaluation of the deformability of PMNs. These differences between the two assays support the use of the agarose assay and the filter technique in parallel in the assessment of the spontaneous movement of PMNs.

There are at least two types of defects involving PMN motility *in vitro*. Firstly, spontaneous movement is impaired, whereas responsiveness to the stimulus is not affected. This results in the 'lazy leucocyte' syndrome (Miller, Oski & Harris, 1971). Secondly, spontaneous movement is not affected, but the response to a stimulus is impaired, as demonstrated with cord PMNs in the present study. Both defects result in poor stimulated movement *in vitro*, and they may also contribute to a poor accumulation of PMNs at the site of infection and inflammation *in vivo*. It appears that any clinical signs caused by a defect in spontaneous movement would most probably be very similar to those caused by a defect in the responsiveness of PMNs. The identification of the defect in PMN motility is interesting, although its clinical significance remains to be seen.

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