Evaluation and comparison of neutrophil bipolar shape formation with a migration assay

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Abstract

The neutrophil shape change response to a chemotactic formylpeptide was assessed. Neutrophil bipolar shape formation (BSF) was also simultaneously assessed with a Boyden chamber-based neutrophil migration assay. Both assays were precise and relatively reproducible; the average coefficient of variation for the BSF assay was 9.6% and 9.2% for the migration assay. In a blind study the BSF assay showed 100% sensitivity at detecting subjects with known abnormal neutrophil migration. Unlike the migration assay, the BSF assay does not require isolated neutrophils, reducing possible cell activation and monitoring the cell response under more physiological conditions. Small blood samples—1 ml or less compared with 20–40 ml for the migration assay—are used, and the method is technically simple. Results are available within 40 minutes, and routine (EDTA) blood samples are used.

It is concluded that the BSF assay is a suitable motility screening test for both the clinical and pharmacological examination of the movement of polymorphonuclear leucocytes.

The ability of polymorphonuclear leucocytes (PMN) (referred to as neutrophils) to migrate directionally in response to chemotactic signals is vital for the rapid and precise extravascular accumulation of these cells. Because of the importance in host defence of neutrophil function, numerous specialised laboratory investigations have been devised.^{1–3} Generally, these tests require the isolation of neutrophils, and although several isolation methods have been developed,³ they are all a potential source of neutrophil activation,² which may influence in vitro behaviour. All neutrophil migration assays tend to be time consuming; there is little agreement about the best investigative regimen, and technical standardisation is poor. Because of their complexity such migration assays have not generally been adopted for routine use.

A simple screening test, however, is available,⁴ which permits a rapid assessment of neutrophil responsiveness. The test monitors the change in neutrophil shape in response to formyl peptides added directly to whole blood and incubated at 37°C. The response is assessed by counting the resultant percentage of "bipolar" neutrophil forms present on Roman-

owsky stained films (fig 1). As neutrophils change shape during movement it has been suggested that the neutrophil bipolar shape formation (BSF) response is functionally related to the locomotor capacity of neutrophils.⁴ An obvious disadvantage of this technique is that the actual movement of cells is not studied. The BSF assay, however, may reflect neutrophil activity similar to that seen with migration assays, implying that the technique is suitable as a motility screening test.

The aim of this study was to compare neutrophil migration, as assessed by a modified Boyden chamber technique,⁵ and neutrophil responsiveness, as assessed by the BSF assay.⁴

Methods

Blood for the BSF assay was collected into EDTA (1.5 mg/ml), maintained at room temperature, and used within one hour of collection. Blood for the migration assay was collected into preservative free heparin (10 µl/ml) and used within two hours of collection. Leucocyte suspensions containing over 90% neutrophils were prepared as described previously.⁶ The cells were suspended in Hanks's balanced salt solution (HBSS), pH 7.4, at a concentration of $5 \times 10^9/l$. Normal studies were performed on 50 blood samples collected from healthy blood donors and volunteers. Blood was also collected from 25 inpatients with rheumatoid arthritis ($n = 8$), diabetes ($n = 5$), liver cirrhosis ($n = 4$), pneumonia ($n = 3$), and those taking corticosteroids ($n = 5$). The samples for both assays were investigated "blind", control and patient samples being collected by venesectors and identified after the assays were performed.

N-formyl-methionyl-leucyl-phenyl-alanine (FMLP; Sigma F-3506) (5 mg) was dissolved in 1.14 ml dimethyl sulfoxide (Sigma D-5879), equivalent to 10^{-2} M FMLP, and aliquoted and stored at -70°C . Working solutions were further diluted in HBSS, as required, to a concentration of 10^{-8} M FMLP.

BIPOLAR SHAPE FORMATION (BSF) ASSAY

Tests were performed by adding 0.1 ml 10^{-8} M FMLP to 0.9 ml whole blood anticoagulated with EDTA. The mixture was gently mixed and allowed to incubate at 37°C for 20 minutes. After incubation and brief remixing two blood films were made, fixed, stained with May-Grünwald Giemsa stain and examined using a $\times 50$ oil objective. Fifty neutrophils from each film were classified according to shape. Negative controls were prepared by adding

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Figure 1a Normal spherical neutrophil as generally seen during routine examination of blood films.

Figure 1b Bipolar neutrophil, as seen after 20 minutes of incubation with $0.1 \text{ ml } 10^{-8} \text{ M FMLP}$.

Figure 1c Amorphous neutrophil after incubation with FMLP, suggesting that the cell is in the early stages of transition to the fully stimulated bipolar form.

0.1 ml HBSS to 0.9 ml EDTA whole blood and proceeding in the same manner as in the test.

Neutrophil shape categories

Spherical neutrophils: Normal neutrophil appearance as generally seen during routine examination of blood films, (fig 1a).

Bipolar neutrophils: These cells have an elongated and constricted appearance, with a distinct, prominent leading end consisting of

light blue agranular cytoplasm. A distinct uropod may be present, (fig 1b).

Amorphous neutrophils: Cells which appear mildly stimulated, with irregular borders showing one or more cytoplasmic projections, suggesting the cell is in the early stages of transition into the fully stimulated bipolar form, (fig 1c).

During this study, the neutrophil BSF results were expressed as the mean percentage number of bipolar neutrophil forms present on two blood films when 50 neutrophils from the central region of each film were so classified. Alternatively, absolute responsiveness values may be calculated (absolute neutrophil count $\times$ percentage of bipolar neutrophil forms).

NEUTROPHIL MIGRATION ASSAY

Chemotaxis was assessed using the method of Jayaswal *et al*⁵ (fig 2). Briefly, vessels containing the leucocyte suspensions were placed on top of $3.0 \mu\text{m}$ pore filters (Millipore (UK) Ltd, Middlesex SSWP, 019, OR), which in turn were placed on top of absorbent discs soaked in chemoattractant (10^{-8} M FMLP). Neutrophils migrate towards the chemotaxin and squeeze through the pores of the filter; these were subsequently fixed and stained. Chemotaxis was then expressed as the "leading front"—the furthest distance two cells have migrated. Duplicate leading front measurements were made and the average taken.

Statistical analysis was performed using the Statgraphics statistical software package (STSC Inc, Rockville, Maryland USA) on an Amstrad PC 1512 microcomputer.

Results

The normal range for both the test samples (FMLP) and negative controls (HBSS) was determined by performing BSF assays on 50 normal subjects. The results given in table 1 show that the percentage bipolar forms present in negative controls did not exceed 3%, while the normal range for the FMLP stimulated samples was 40% to 61% bipolar forms (mean $\pm 2\text{SD}$).

The normal range for both the test samples

Table 1 Statistical analysis of normal BSF results

	Negative control (HBSS)	Test (FMLP)
n =	50	50
Mean (SD)	1.5 (0.6)	50.6 (5.4)

Figure 2 Schematic diagram of the multi-well chemotaxis plate.

Table 2 Statistical analysis of normal migration leading front measurements

	Negative control (HBSS)	Test (FMLP)
n =	50	50
Mean (SD)	28.0 (3.3)	95.8 (4.2)

Table 3 Reproducibility of both techniques

Bipolar shape formation	Sample		
	A	B	C
n =	20	20	20
Mean	58.6	54.3	44.6
Range	59–64	43–65	37–57
SD	3.4	4.7	5.5
CV	5.1	10.4	13.3
Migration assay			
n =	20	20	20
Mean	91.5	98.0	92.2
Range	90–100	89–103	93–104
SD	6.9	10.7	8.4
CV	7.5	10.9	9.1

(chemotaxis towards 10^{-8} M FMLP) and negative controls (chemotaxis towards HBSS) was determined by performing migration assays on 50 normal samples (table 2). The results indicate that the normal range for the negative controls (HBSS) was 21 to 35 μ m, while that for the test samples was 87–105 μ m (mean $\pm$ SD).

The reproducibility of both techniques was assessed by performing 20 serial assays on each of three normal samples (table 3). Statistical analysis showed coefficients of variation (CV) for the BSF assay ranging from 5.1%–13.3%, while the CVs for the migration assay ranged from 7.5–10.9%.

Simultaneous BSF assays and neutrophil migration assays were performed on 50 normal controls and 25 inpatients. Table 4 shows the mean and standard deviation of the data, as well as their spread, for each group. The control group showed both a normal mean percentage BSF and mean leading front value, while both parameters were significantly ($p < 0.001$) reduced in the patient group. This mean reduction in values for both parameters was also observed for all subjects in the patient group. Furthermore, BSF and migration assays were significantly ($p < 0.01$) correlated in the patient group. Thus patients with abnormal leading front results also showed abnormal BSF results. Conversely, a normal leading front measurement always corresponded to a normal BSF result. Linear regression analysis of the BSF and leading front for the control group gave a CV of 0.058 ($p > 0.1$), while that obtained for the patient group was 0.552 ($p < 0.01$) (fig 3).

Table 4 Bipolar shape formation (BSF) and leading front (LF) measurements in control and patient groups

	Controls	Patients
n =	50	25
Mean BSF	50.7	20.7
Range	44–59	8–39
SD	5.4	9.2
Mean LF	94.9	69.0
Range	87–104	45.5–85.5
SD	3.4	10.8

Figure 3 Linear regression analysis of the bipolar shape formation and migration assay for the control and patient groups.

Discussion

Most neutrophil migration assays depend on purified neutrophils, which requires large volumes (20–40 ml) of blood. Isolation of neutrophils may take one to three hours and is a potential source of cell activation in its own right.² Migration assays are also time consuming, labour intensive, and require specialist technical ability and equipment. The numerous methodologies available mean that results vary from laboratory to laboratory. Most investigators also experience diurnal fluctuations for unknown reasons.⁷ This makes it necessary to include a “healthy” control each time and to express the results relative to the controls,⁸ increasing the number of samples tested. Furthermore, slight variation within a single test system may substantially modify the results.⁹ These difficulties may account for the low adoption rate of classic migration testing.

A motility screening test,⁴ however, has been available for some time. The method eliminates the requirement for isolating neutrophils, so reducing cell activation, and therefore monitors the neutrophil response under less artificial conditions. Small blood samples (1 ml or less) are required, making it suitable for use with infants and small children.¹⁰ Results can be available within 40 minutes, specialist equipment is not required, and the test may be performed on routine (EDTA) blood samples. The present study describes a method for the neutrophil bipolar shape formation assay, and establishes normal values—less than 3% bipolar neutrophil forms in negative controls (HBSS), and 40–61% bipolar forms in normal FMLP stimulated samples. This range is wider than the 35% to 45% BSF response reported in the original method,⁴ and lower than that reported by others (65% to 75% BSF response).¹⁰ A possible explanation for this discrepancy may be the different experimental procedures used by the various workers. For instance, Jadwin *et al* used N-formyl-methionyl-phenylalanine (FMP) as the “stimulant” with incubation at room temperature and 37°C for various time intervals up to 40 minutes.⁴ Davis *et al* used FMLP to stimulate bipolar

shape formation and incubated their reaction mixtures at room temperature for 30 minutes,¹⁰ while a 20 minute incubation at 37°C using 10^{-8} M FMLP was used throughout this study. No difference, however, was reported for the negative controls (HBSS) in the respective studies. These results emphasise the need both for each laboratory to establish its own reference range and for the method to be standardised.

Problems associated with manual leucocyte differential counts,¹¹ such as the difficulty of obtaining an even distribution of leucocytes in the film and the random error in establishing the proportions of cell types, also apply to the BSF assay. Considering this limitation, the CVs observed (5.1%, 10.4%, and 13.3%, respectively) were reasonable. These discrepancies might be reduced by the use of a blood "spinner" (Larc, Corning Medical, New York, USA) to prepare blood films, as described in the original method⁴ and subsequent reports.¹⁰

We found that the BSF method was technically simple to perform and gave relatively good reproducibility as does the more complicated migration assay in our hands; the average CV for the BSF assay was 9.6% and 9.2% for the migration assay. As expected, all the samples in the control group, for both bipolar shape formation and migration assays, gave values within their respective normal ranges. An apparently poor correlation ($r = 0.058$) between the two techniques was obtained in the control group. This may be explained by the lack of "scatter" of results, which all fell within the two narrow normal ranges, (fig 3). The patient group showed better correlation between the BSF and migration assays ($r = 0.552$). These samples all gave abnormal results for both tests, however, showing a greater scatter of results and contributing to the better correlation (fig 3).

Statistical analysis showed a significant difference between the control and patient groups for both the leading front and bipolar shape formation assays ($p < 0.001$). Moreover, in the blind study all samples showing abnormal leucocyte migration showed similarly abnormal BSF responses. In other words the neutrophil bipolar shape formation assay showed complete sensitivity at detecting samples with abnormal migration; similarly, normal cell migration always corresponded to a normal BSF result.

Neutrophils tested immediately before the BSF and migration assays were judged to be greater than 95% viable by trypan blue exclusion. Migration assays "select" the most active motile cells from a population; the BSF assay reviews a random sample of cells, small changes in viability could therefore affect BSF results. Although this was not encountered during the current work, it might be advisable to assess viability along with BSF assays.

In certain diseases such as rheumatoid arthritis the circulation may be depleted of the most motile cells; alternatively a wider range of cell activation may reduce the number of cells able to migrate. Results from migration assays, which "select" neutrophils making the greatest

progress through the filter, must therefore be interpreted with care. The "average" value derived from the BSF test, however, may also be susceptible to such sampling errors. Further comparisons with skin windows¹² or using mobilised granulocytes¹³ might provide more information, if this was thought to be a problem.

The BSF assay, therefore, has qualities which make it suitable as a motility screening test for use by non-specialised laboratories in preference to migration assays. Such a screen could be used to detect patients who might benefit from further detailed investigation. This screening test is also suitable for studies involving multiple simultaneous samples, such as the dose-effect of drugs,¹⁴ the assessment of changed neutrophil function in disease groups,¹⁵ or familial studies. The BSF response also occurs without apparent dependence on membrane deformability or adherence to surfaces and is said to precede the final locomotive or chemotactic response.¹⁶ It may therefore provide valuable information on whether an intrinsic cell defect is related to the early "sensory" and functional events of neutrophil activation, rather than the later events of actual locomotion. The BSF assay seems to be sensitive to the same clinical stimuli as migration assays, has about the same degree of performance errors due to biological or technical variants, and is much easier to perform. For these reasons we believe it worth pursuing as a screening test for neutrophil locomotor function.

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