Polymorphonuclear Motility: Measurement by Computer-Linked Image Analysis

By G. B. Howe, K. V. Swettenham, and H. L. F. Currey

Most methods of measuring neutrophil motility provide information mainly about the performance of a small proportion of the fastest moving cells. Application of a computer-linked image analysis technique, using the "Quantimet," provides a convenient, automated method of measuring the motility of the whole cell population. This makes it possible to test whether changes in motility represent a homogeneous alteration affecting all cells or a change in the numbers or performance of a subset of cells. In this study the neutrophils from patients with uncomplicated rheumatoid arthritis were found to perform similarly to normals, while cells from patients with Felty's syndrome were markedly slower. This was an overall, homogeneous slowing of the whole cell population, not due to a loss of fast moving cells.

Following the original description by Boyden, most assays of neutrophil motility have been based on the movement of cells through Micro-pore filters. The original method involved counting the numbers of cells reaching the distal surface. This is now recognized as fallacious because cells reaching this surface tend to drop off. Zigmond and Hirsch overcame this problem by employing a shorter incubation time (so that the cells did not reach the distal surface) and measuring the distance to the "leading front": the most distal point at which two cells were in clear focus. This is presumed to represent the distance travelled by the fastest moving cells but more literally, measures the vertical distance travelled by those cells that have moved furthest from the top of the filter. More recently, Maderazo and Woronick counted the numbers of cells at specified levels through the filter and derived a "leukotactic index," which reflects the mean distance travelled by all cells. When the neutrophils of normal subjects are tested, the results by this rather laborious technique correlate well with the leading front method.

Both the leading front measurement and the leukotactic index provide a somewhat crude measure of neutrophil motility; they may detect an abnormality but they will not differentiate between an overall alteration affecting all cells and a change in the numbers or performance of a subpopulation. Recent studies point to there being functional variations between subsets of neutrophils.

In order to detect possible subpopulation differences, it is necessary to determine the distribution of all cells through the filter. Done manually, this is excessively time consuming and laborious. We have therefore developed an automated technique using computer-linked image analysis.

To illustrate the application of this technique, we have used data that form part of a larger study of neutrophil functions in rheumatic diseases.

MATERIALS AND METHODS

Patients
Sixteen healthy laboratory and clinic staff were used as controls. Seven patients with rheumatoid arthritis (RA) complicated by persisting neutropenia and splenomegaly (Felty's syndrome) and six patients with uncomplicated RA were also studied. The patients were taking only analgesics with or without nonsteroidal antiinflammatory drugs.

Neutrophil Migration
A modification of the method of Mowat and Baum was used. Polymorph-enriched buffy layer prepared from heparinized whole blood using methyl cellulose (mean, 79% neutrophils) was centrifuged (Shandon cytocentrifuge). Twenty g for 5 min) on the Micro-pore filters of 3-μm pore size (Schleicher and Schull, Dassel, West Germany). Thirty randomly selected filters ranged in thickness from 181.6 to 215.9 μm (mean 195.4 μm) when dry. When wet, this thickness increased by up to 50%. The numbers of cells centrifuged on the filter were not standardized, but appropriate experiments established that variations of numbers within the range of these experiments did not significantly affect the outcome. The filter with its cell pellet on the top surface was then secured in a modified Sykes-Moore chamber. To assess random motility Hanks' balanced salt solution (HBSS), buffered to pH 7.2 with HEPES, was placed in the two compartments (above and below the filter). For directed motility (chemotaxis) HBSS containing 33% serum "activated" by 5% casein (BDH Chemicals, England) was placed in the lower chamber. The cell motility data used as illustrations in this paper all relate to directed migration using serum activated by casein as attractant. After incubation at 37°C for 20 min, filters were fixed overnight in SUSa solution (mercuric chloride 4.5 g, sodium chloride 4.5 g, trichloro-acetic acid 2 g, 40% formaldehyde solution 20 ml, glacial acetic acid 4 ml, distilled water 80 ml), stained with hematoxylin, dehydrated in absolute ethanol, cleared in xylene, then mounted in immersion oil (Fractoil, Raymond and Lamb, London). The relatively short incubation time of 20 min was used to ensure that no cells traversed the full thickness of the filter.

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occupied by cells and hence of cell numbers. The sensitivity of the output from the camera is displayed on a screen and is simultaneously fed into a densitometer that measures specified levels of greyness. With appropriate staining, the densitometer “sees” the cells as numbers of picture points of specified grey level, thus providing a measure of the proportion of the microscopic field occupied by cells and hence of cell numbers. The sensitivity of the instrument is set so that it “sees” the whole of each cell that is in focus and virtually nothing that is out of focus. This depth of focus is between 4 and 8 μm. The output from the densitometer is fed into a linked computer for storage and data manipulation.

Duplicate filters were employed. Using a ×40 objective lens, the microscope was focused first on to the layer of cells on the upper surface of the filter. An attachment to the fine focus control then provided stepwise lowering of the focal plane by 10–μm steps, a reading being taken at each level through the filter. Given a refractive index of approximately 1.50 for the various materials in this optical system, this is equivalent to taking a reading every 15 μm through the actual filter. Five such optical “cores” were measured on each of the duplicate filters. Cell density estimates at each 15-μm level were thus based on the mean of 10 independent readings. These data made it possible to construct a profile of cell distribution through the thickness of the filter. They were also used to calculate the leukotactic index (see below.)

Leading Front Measurements

The 2-cell leading front measurement was determined by the method of Zigmond and Hirsch, using a ×100 oil immersion objective and 5 random readings from each of 2 replicate filters.

Analysis

The Quantimet data were used to calculate a leukotactic index (LI), the formula of Maderazo and Woronick being modified to apply to cell areas instead of cell numbers (readers are referred to the original paper for the theory behind the formula). Thus:

\[
LI = \frac{\sum \text{Area of cells at each level (μm}^2\text{)} \times (\text{True depth from surface } - 15 \text{μm})}{\text{Area of all cells (μm}^2\text{)}}
\]

After centrifugation of the cells onto the filter, and before incubation started, the so-called “monolayer” (m) of the cells on the upper surface was 20–30 μ deep, with approximately 50% of the cells occupying the first 15 μ of the preparation. At the end of the incubation some cells remained in this “monolayer.” The thickness of the “monolayer” was therefore included in the formula for greater accuracy. The modified formula thus assumes that the starting point (zero level) is 15 μ below the top surface of the preparation. The modified formula thus becomes:

\[
LI = \frac{\sum \text{Area of cells at each level (μm}^2\text{)} \times (\text{True depth from surface } - 15 \text{μm})}{\text{Area of all cells (μm}^2\text{)}}
\]

This is the modified LI + m (+ m indicating that the monolayer is included in the calculation), representing the mean distance travelled by all cells. LI – m (excluding the monolayer) represents the mean distance travelled by the migrating cells. The modified formula to calculate LI – m becomes:

\[
LI = \frac{\sum \text{Area of cells at each level (μm}^2\text{)} \times (\text{True depth from surface } - 15 \text{μm})}{\text{Area of all cells (μm}^2\text{)}}
\]

Although slightly different values are therefore obtained, the overall conclusions remain the same whether modified or unmodified formulae are used. Modified data are used throughout this article.

RESULTS

Table I gives the result of the leading front measurements and leukotactic indices for the three groups studied. Controls and uncomplicated rheumatoids do not differ significantly. However, the Felty’s patients are significantly slower than both the controls and the uncomplicated rheumatoids.

Both leukotactic indices correlated well with the leading front measurement. Figure 2 shows the relationship of LI – m to leading front for all subjects tested.

The leading front measurements establish that in Felty’s syndrome the fastest moving cells travel less far than cells in uncomplicated RA or controls, while the leukotactic index shows that the mean distance travelled per cell is less in Felty’s. Neither measurement establishes whether the differences represent an overall slowing of the whole population of cells or whether it is due to a depletion, or slowing, of a fast

### Table 1. Leukotactic Index (LI) and Leading Front (LF)

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<th>Measurements for Directed Migration in Control Subjects, Uncomplicated Rheumatoids, and Patients With Felty’s Syndrome.</th>
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<td>LI + M (μm)</td>
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<tr>
<td>LI – m (μm)</td>
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<td>Leading Front (μm)</td>
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See Materials and Method for explanation of calculations.

Results are means ± SD. Differences by Student’s t-test.

*Significantly different from controls (p < 0.001) and RA (p < 0.01).
†Significantly different from controls (p < 0.01) and RA (p < 0.02).
‡Significantly different from controls (p < 0.001) and RA (p < 0.05).
moving subpopulation. For this it is necessary to consider the profiles of cell distribution derived from the image analysis data.

Figure 3 shows the profiles of cell distribution in the three groups at the end of 20-min incubation. The controls and uncomplicated rheumatoids are not significantly different, while the Felty’s cells are clearly slower. To test whether this slowing affected all the cells equally, the cumulative percentage of total cell area was plotted against distance for each group (Fig. 4). From these curves it is possible to read off the depth distribution of various percentiles of the total cell population. This allows cell populations to be compared by plotting these depth readings for each percentile in one group against the depth reading for the same percentile in another group. Figure 5 shows this for controls versus uncomplicated RA and Felty’s versus controls. The former produces a near straight line plot with a slope of about 45°, indicating that the two populations are homogeneous and have approximately identical distribution of cell speeds. The plot of Felty’s versus controls yields a near straight line with a slope of 22°, indicating that the Felty’s cells are traversing at about half the speed of control cells, and that this is an overall slowing of a homogeneous population. Had there been, for example, a selective depletion or slowing of a fast moving subset of neutrophils, a curved line would have resulted. The plot for Felty’s versus uncomplicated rheumatoids (not shown in Fig. 5) gives an almost identical straight line.

**DISCUSSION**

Micropore filters have stood the test of time as a convenient medium in which to study rates of neutrophil migration. However, variability in filter thickness and the fact that cells that reach the lower surface tend to fall off23 (an observation we have confirmed) means that counts of cells on this surface are unreliable measures of cell migration. The leading front technique of Zigmond and Hirsch3 overcomes this as the shorter incubation period means that all the cells remain within the thickness of the filter. This technique measures the motility only of the few most rapidly moving cells; the bulk of cells remain much closer to the starting surface (Fig. 2). Nevertheless, in a normal population of cells, this measurement correlates well with the mean distance travelled by all the cells determined by the painstaking technique of Maderazo and Woronick.2 For this reason the leading front method remains a good screening test for detection abnormalities of neutrophil motility.

It has to be remembered that the Micropore filter is a matrix through the “pores” of which the polymorphs creep, more or less along a chemotactic gradient, but by no means rectilinearly. Thus, the frontrunners are not necessarily the “fastest” individuals; they may have been slower, but have travelled more directly. Movements are not necessarily uniform, but may possibly occur in fits and starts. Considerations such as these have to be kept in mind when equating “motility” with the greatest distance travelled in a given time. Also, it is doubtful whether the pattern of cell distribution by itself can differentiate between directed and nondirected motility.

The leading front method cannot differentiate between overall changes affecting all cells and alterations in function or numbers within a subset of cells. Further, recent studies employing rosetting techniques4 suggest that there are subpopulations of neutrophils in the peripheral blood that differ functionally. Thus, the rate of migration of the cells in front may not be representative of the migration of the cell population as a whole. It therefore becomes important to determine the homogeneity of any alteration in
neutrophil motility. In order to do this it is necessary to determine the profile of cell distribution throughout the thickness of the filter. This information allows analysis by methods such as that employed here, in which the percentile distribution of cells by depth is compared between cell populations.

Maderazo and Woronick (2) have shown that the data required for this type of analysis can be obtained by counting cells at different levels by eye. However, this technique is excessively time-consuming. The application of image analysis described here offers a rapid, automated, and reproducible method applicable to large numbers of filters. Further, the linked computer provides simultaneous manipulation of the data. A previous report of automated chemotaxis measurement (7) employed filter surface counts. Reasons have been given why such counts cannot be regarded as reliable.

Our experience has highlighted the importance of some technical details in this method. Immersion oil is preferred to synthetic resin as mounting medium. It penetrates the filter better and gives more even trans-
mission of light. Filters and cells remain well preserved for at least 6 mo.

The widely recognized problem of filter variability, particularly thickness, affects all the techniques of cell motility measurement. Using the Quantimet, these variations lead to variations both in cell staining intensity and transmission of light. Although these problems can be minimized by appropriate adjustments to the sensitivity of the instrument, the search for a more consistent filter is important.

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REFERENCES


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