Observations on the Motile Behavior of Individual Neutrophils From a Patient With Recurrent Bacterial Infections

By Thomas H. Howard, Jerry A. Winkelstein, Min-Fu Tsan, and William H. Zinkham

A technique for determining the velocity of individual neutrophils and a standard chemotactic assay were used to study the motile behavior of neutrophils from a patient with recurrent bacterial infections. A chemotactic assay under agarose showed that the abnormal neutrophils were deficient in nondirected and directed movement and that neither the patient's serum nor plasma was inhibitory to normal neutrophil movement. Microscopic study of individual neutrophils showed that the movement morphology and velocity of the patient's neutrophils were abnormal. Cells from the patient failed to orient or to show cytoplasmic flow. The mean velocity of the patient's neutrophils was 2.6 μm/min (controls 11.9), and the range was 0–7.0 μm/min (controls 2.0–21.0). The velocity of normal neutrophils in the patient's plasma was not decreased and the velocity of the patient's neutrophils was not increased by ascorbic acid, carbamylcholine, or dibutyl cyclic GMP. While the cause of decreased neutrophil movement was not defined, no abnormality of neutrophil adherence, glucose oxidation, or actin polymerization was found. The results of this study indicate that observation of individual cells to determine the morphology and velocity of cell movement may enhance our understanding of factors that affect neutrophil movement.

During the past 20 years studies on patients with increased susceptibility to infection have led to the description of many new disorders of neutrophil function. Initial reports showed abnormalities in the ability of cells to kill bacteria. More recently, defects in cell movement have been recognized as a cause of increased susceptibility to infections. Most movement disorders have been defined by utilizing the Boyden chamber and vertical capillary tube techniques. These methods involve the study of large populations of cells and thus are best suited to identifying major deviations from the normal. However, the spectrum of velocities and movement morphologies of individual cells within the population under study is difficult to determine.

Another method for evaluating neutrophil movement is by microscopic observation of living cells on a glass substratum. Sabin introduced this technique in 1923, but the method was underutilized until recently, when Sabin's original observations were extended to include measurements of the velocities of single cells.

This report describes a patient with recurrent infections associated with...
defective neutrophil movement. By observing individual cells, two defects of neutrophil motility were defined: (1) an abnormality of cell morphology during movement and (2) a marked reduction in the velocity of movement. Results of this study suggest that observation of single leukocytes may offer advantages for defining and classifying leukocyte movement disorders.

CASE REPORT

A black female infant developed *Staphylococcus aureus* omphalitis at 3 days of age. During the ensuing 15 mo of the child's life, *S. aureus* infection recurred at various sites (omphalitis, rectovaginal fistula, cellulitis, deep buttock abscess, pneumonia). No pus was present at sites of infection, and gram-stained smears from sites of infection showed many gram-positive organisms and occasional monocytes but no polymorphonuclear leukocytes.

Peripheral blood findings included anemia (PCV 0.28, hemoglobin 7.9 g/dl), normal platelets (280 x 10⁹/liter), and marked leukocytosis (WBC 108 x 10⁹/liter) with increased absolute counts of neutrophils, lymphocytes, and monocytes (2% metamyelocytes, 4% juvenile, 65% neutrophils, 15% lymphocytes, 14% monocytes). Despite the use of antibiotics and control of infections, the leukocytosis and increased absolute counts persisted throughout the patient's life (Fig. 1). The morphology of the neutrophils and neutrophilic precursors was normal in Wright-stained preparations of the bone marrow and peripheral blood. Leukocyte alkaline phosphatase was elevated (patient's score 305; control 105). Electron microscopy of the neutrophils showed normal morphology.

Blood chemistries, urinalysis, IVP, and erythrocyte enzymes, including G-6-PD, were normal. Levels of immunoglobulins were increased: IgG 2300 mg/dl IgM 279 mg/dl IgA 235 mg/dl, and IgE 2101 mg/dl. B and T lymphocyte function was normal as evidenced by normal antibody titers (anti-A, anti-B, diphtheria, tetanus, polio), normal anamnestic response to diphtheria/tetanus toxoid, and normal ³H-thymidine incorporation in response to allogeneic cells and PHA. Studies of the patient's phagocytes showed normal killing of staphylococcus 502A and normal superoxide production. Only a rare neutrophil appeared in a Rebuck skin window during 24 hr of observation.

MATERIALS AND METHODS

Single-cell technique. Wet preparation of viable granulocytes was made using a modification of the method of Ramsey. All materials used to prepare wet preparations were sterile. Whole blood, 0.4 ml, without anticoagulant was incubated in high humidity at 37°C on a coverslip. After 20 min the clot was washed off the coverslip with 5 ml Veronal buffer pH 7.2. A circle of leukocytes (97% granulocytes, less than 2% eosinophils and basophils) remained adherent to the coverslip. A drop of supporting medium, either fresh heparinized plasma or Kreb's Ringers phosphate buffer with glucose, was placed on a glass slide. The coverslip, with cells attached, was then inverted onto the drop of supporting medium, and the coverslip edges were sealed with vaseline. Observations were made using a Leitz light microscope at 450x. Position coordinates of individual cells were taken each minute for 10 min with an ocular micrometer, and the path of migration was plotted to scale on graph paper. The total distance moved in 10 min was mea-
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Secured and the velocity expressed as μm moved/min. Only the velocities of pseudopod-forming cells were measured.

Utilizing this technique the day-to-day variation of the mean velocity of normal neutrophils was less than 10%. The velocities determined for normal neutrophils and the patient's neutrophils remained the same during 3 hr of observation. The time required to complete a single assay was 1.5 hr. At the end of 3 hr observation greater than 95% of the cells remained viable as determined by trypan blue exclusion.

Chemotaxis under agarose. Chemotaxis was measured under agarose by the method of Nelson et al. The incubation time for studying neutrophils in mixed leukocyte preparations was 8 hr; purified neutrophil assays were incubated for 4 hr. Cell movement was quantitated by measuring the linear distance from the edge of the cell well to the advancing edge of migrating neutrophils with a Bausch & Lomb AVB 73 microscope and a calibrated eyepiece micrometer.

Preparation of leukocytes. Leukocyte-rich plasma was separated from heparinized whole blood (10 U/20 cc) by sedimentation with 17.5 ml Dextran solution (10 parts Dextran 60:1 part D5W) per 50 ml whole blood and was centrifuged at 200 g for 15 min. The pellet of mixed leukocytes was washed twice in 40 vol Minimal Essential Medium (MEM) (Gibco) and resuspended at a concentration of 40 x 10^6 WBC/liter.

To obtain pure granulocytes, mixed leukocytes were separated as above, resuspended in 40 vol 0.83%, NH₄Cl, and incubated at 4°C for 30 min to lyse red cells. The red cell-free mixed leukocytes were washed in 40 vol MEM and resuspended in 10 ml MEM. The 10 ml of mixed leukocytes was gently layered onto 10 ml Ficoll-Hypaque mixture according to the method of Böyum and centrifuged at 450 g for 40 min at 25°C. The pellet that contained 95% granulocytes was washed twice in 40 vol MEM and resuspended to a concentration of 25 x 10^6 WBC/liter for study.

The chemotactic factors used were an Escherichia coli culture filtrate (kindly supplied by Dr. Paul Quie) and zymosan-activated serum. Zymosan-activated serum was prepared by incubating 50 mg washed zymosan (Sigma) in 1 ml serum for 30 min at 37°C. Some zymosan-activated serum was standardized by activating a single batch of serum from one individual; aliquots were stored at -70°C. Each newly thawed aliquot was tested with a previously tested control to assure day-to-day reproducibility.

Inhibitor studies were done with normal mixed leukocytes. The normal cells were preincubated in the patient's fresh serum or heparinized plasma for 2 hr at 37°C. The cells were centrifuged resuspended in MEM, and assayed.

Studies of leukocyte adherence, metabolism, and actin. Granulocyte adherence was studied by the method of MacGregor et al. Glucose oxidation by granulocytes was determined by the method of Tsan et al. The percentage polymerizable leukocyte actin was determined according to the method of Boxer et al.

Controls for the chemotactic assay under agarose included healthy adults, two children (ages 4 and 8 mo) with S. aureus abscesses and leukocytosis, two healthy black children (ages 6 and 9 mo). Controls for the direct visual assays were healthy adults.

RESULTS

Evaluation of neutrophil movement by chemotaxis under agarose. A chemotactic assay under agarose was used to study the ability of the patient's serum to generate chemotactic activity and the ability of the patient's neutrophils to move. The patient's zymosan-activated serum attracted cells from two normal individuals to the same degree as normal zymosan-activated serum (Table 1), indicating that the patient's serum could generate chemotactic activity.

<table>
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<tr>
<th>Table 1. Ability of the Patient's Zymosan-Activated Serum (ZAS) to Attract Normal Neutrophils</th>
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<tr>
<td>ZAS Source</td>
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<td>Controls</td>
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<td>Patient</td>
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Mean ± 1 SD moved by neutrophils from two normal individuals toward zymosan-activated serum. Number of experiments in parentheses.
Studies on the patient's neutrophils showed that the nondirected and directed movement of these cells were decreased. In the absence of chemotactic factors, normal cells moved a greater distance (mean 0.95 mm) beyond the cell well than did the patient's cells (mean 0.45 mm) (Fig. 2A). As shown in Fig. 2B, in the presence of normal serum activated with zymosan the patient's cells moved a mean distance of 0.70 mm toward the well containing chemotactic factor, whereas normal cells moved 1.95 mm. The patient's cells showed the same defective directed movement toward zymosan-activated serum from three normal individuals. In addition, the patient's cells moved only 0.50 mm toward an E. coli culture filtrate (control 1.60 mm). Purified preparations of neutrophils showed the same abnormalities in cell movement; the movement of normal cells was not affected by prior exposure to the patient's fresh heparinized plasma or serum (see Table 2).

Repeated assays over a period of 6 mo showed persistence of the abnormality of nondirected and directed movement. The defect was unrelated to the presence of acute infection, the level of the white cell count, or the administration of antibiotics.

Movement morphology and velocity of single neutrophils. The morphology and rate of neutrophil movement in anticoagulated plasma were determined

<table>
<thead>
<tr>
<th>Plasma or Serum Source</th>
<th>Incubation Medium</th>
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<tr>
<td></td>
<td>MEM</td>
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<tr>
<td><strong>Directed movement</strong></td>
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<tr>
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<tr>
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<td>2.11</td>
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<tr>
<td><strong>Nondirected movement</strong></td>
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<tr>
<td>Control</td>
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<td>Patient</td>
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*Millimeters moved by normal neutrophils toward zymosan-activated serum after incubation in MEM, plasma, or serum. Mean of two experiments.

†Millimeters moved by normal neutrophils in the absence of zymosan-activated serum after incubation in MEM, plasma, or serum. Mean of two experiments.
by microscopic observation of single neutrophils. The sequence of morphologic changes that occur during the movement of normal cells includes adherence, pseudopod formation, orientation, and cytoplasmic flow. The patient’s neutrophils adhered and formed multiple thin pseudopodia or thick cytoplasmic bulges, but orientation and cytoplasmic flow were rarely observed. The patient’s cells in normal plasma continued to show abnormal movement morphology, and the movement morphology of normal cells was unchanged when studied in the patient’s plasma.

Neutrophil velocities on 172 cells from ten normal individuals (five female, five male) and 67 cells from the patient were measured. As shown in Fig. 3, velocities of normal neutrophils follow a normal distribution with a range of 2.0–21.0 μm/min (mean 11.9). In contrast, the patient’s mean neutrophil velocity was 2.6 μm/min (range 0–7.0), and the frequency distribution of velocities was asymmetric. The velocity of cells that failed to adhere or form pseudopodia were not determined. Only 20% of the patient’s cells formed pseudopodia, while 80%–90% of control cells formed pseudopodia. Consequently, the velocity profile of the patient’s neutrophils may exclude a large number of immobile cells.

The velocity of the patient’s neutrophils remained the same in autologous plasma or Kreb’s-Ringer phosphate buffer with glucose, and it was not enhanced by incubation in 5–10 mM ascorbic acid, 1 mM dibutyril cyclic GMP, or 8 mM carbamylcholine. Also, the patient’s plasma had no effect on the velocities of normal neutrophils.

**Other studies.** Other investigations to define the reason for the abnormality in neutrophil movement included studies of neutrophil adherence, metabolism, and actin. Using the method of MacGregor et al., 85% of the patient’s peripheral blood granulocytes adhered to columns of nylon fibers (control 85%).

Metabolically, when neutrophils were exposed to 14C-1- and 14C-6-labeled glu-

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**Fig. 3.** Histograms of neutrophil velocities determined at 37.0°C in autologous plasma. (A) Neutrophils from normal subjects (n = 172, 15–20 neutrophils from each of ten subjects). (B) Neutrophils from patient (n = 67 from assays done during period of 4 mo).
Table 3. Glucose Oxidation by Neutrophils

<table>
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<th>Phagocytosing</th>
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<tr>
<td>1-14C-glucose</td>
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<tr>
<td>Patient</td>
<td>13.7</td>
<td>75.6</td>
</tr>
<tr>
<td>Controls</td>
<td>19.1 ± 3.5 (9)</td>
<td>66.2 ± 6.4 (9)</td>
</tr>
<tr>
<td>6-14C-glucose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient</td>
<td>0.33</td>
<td>1.24</td>
</tr>
<tr>
<td>Controls</td>
<td>0.52 ± 0.24 (7)</td>
<td>0.92 ± 0.30 (7)</td>
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Results expressed as nmoles 14CO2 released/hr/10^7 PMN (mean ± 1 SEM). Number of experiments in parentheses.

cose at rest or during phagocytosis, they produced normal amounts of 14CO2 (Table 3).7 Actin extracted from the patient’s leukocytes was 85% polymerizable in 100 mM KCl at 25°C (normal 80%–95%). The quantity of actin in the patient’s leukocytes was not determined.

DISCUSSION

This paper describes a technique for studying the motile behavior of individual neutrophils and its application to the investigation of neutrophils from a patient with recurrent bacterial infection. A chemotactic assay under agarose showed that the patient’s cells were deficient in directed and nondirected movement. The single-cell technique complemented the chemotactic assay by allowing quantification of the velocity of individual neutrophils and a description of the movement morphology of the abnormal cells.

Methods currently available for studying neutrophil movement involve observations on large populations of cells. Variations in the movement morphology and velocity of individual cells may escape detection by such methods. In contrast, by recording the velocities of individual cells one can recognize the spectrum of neutrophil velocities and may define subpopulations of cells with differing motile behavior. For example, the nondirected movement of neutrophils from neonates is decreased in the Boyden chamber and normal in the vertical capillary tube technique.12 This difference is presumably due to decreased deformability of the neutrophils. Is this deformability reflected in the cells’ velocities? Do some individuals, like neonates, have a mature and an immature population of neutrophils with differing movement characteristics? Measurement of mean cell velocity on masses of cells have not answered these questions. However, observing and quantifying the movement of single cells might.

Problems with the single-cell technique utilized in this study include the following: (1) uncertainty as to what type of velocity is being measured—basal, stimulated, or chemotaxed—and (2) cells that do not adhere or form pseudopodia are excluded from the study. We cannot be certain which velocity was measured in these studies. However, two observations suggest that the velocities were basal. First, by plotting the coordinates taken on individual neutrophils during movement one can reproduce the path followed by the migrating cells and determine the velocity and direction of movement. No directional component of neutrophil migration was found, thus indicating that no gradient of chemotactic factors existed. Second, since the velocities of all neutrophils
studied remained the same when either Kreb's-Ringer phosphate buffer with glucose or autologous plasma was used as supporting medium, it is unlikely that a plasma-derived chemotactic factor was stimulating the neutrophils to exceed their basal velocity. These observations suggest but do not prove that the reported velocities are those of nonstimulated, nonchemotaxed neutrophils.

Since the velocities of only adherent, pseudopod-forming cells were determined in our studies, the results may not be representative of all of the circulating neutrophils. Such a sampling bias probably overestimates the velocity of neutrophils and would not explain the decreased velocity of the patient’s neutrophils.

Only a few patients with decreased nondirected movement of neutrophils have been described in the literature.11,13,17 The movement morphology of the abnormal cell was reported in only one case. Boxer et al.11 studied a patient whose leukocytes contained decreased amounts of polymerizable actin. The neutrophils of their patient “remained relatively immobile, spread poorly on glass and extended a few fork-like pseudopodia.”11 In contrast, our patient's neutrophils contained normal amounts of polymerizable actin and formed abnormally thin pseudopodia or thick cytoplasmic bulges. Most of the abnormal cells failed to orient, and cytoplasmic streaming was not observed.

The molecular basis of the defective neutrophil movement in this patient is unknown; however, the abnormal neutrophil movement is clearly described and quantitated by the study of individual cells. Similar characterization of the motile behavior of neutrophils from patients and normals could offer valuable insight into the mechanisms of neutrophil movement.

ACKNOWLEDGMENT

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REFERENCES

11. Boxer L, Hedley-White T, Stossel T: Neutrophil actin dysfunction and abnormal


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