STRUCTURAL ANALYSIS OF HUMAN NEUTROPHIL MIGRATION

Centriole, Microtubule, and Microfilament Orientation and Function during Chemotaxis

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ABSTRACT

Orientation of nucleus, centriole, microtubules, and microfilaments within human neutrophils in a gradient of chemoattractant (5% *Escherichia coli* endotoxin-activated serum) was evaluated by electron microscopy. Purified neutrophils (Hypaque-Ficoll) were placed in the upper compartment of chemotactic chambers. Use of small pore (0.45 μm) micropore filters permitted pseudopod penetration, but impeded migration. Under conditions of chemotaxis with activated serum beneath the filter, the neutrophil population oriented at the filter surface with nuclei located away from the stimulus, centrioles and associated radial array of microtubules beneath the nuclei, and microfilament-rich pseudopods penetrating the filter pores. Reversal of the direction of the gradient of the stimulus (activated serum above cells) resulted in a reorientation of internal structure which preceded pseudopod formation toward the activated serum and migration off the filter. Coordinated orientation of the entire neutrophil population did not occur in buffer (random migration) or in a uniform concentration of activated serum (activated random migration). Conditions of activated random migration resulted in increased numbers of cells with locomotory morphology, i.e. cellular asymmetry with linear alignment of nucleus, centriole, microtubule array, and pseudopods. Thus, activated serum increased the number of neutrophils exhibiting locomotory morphology, and a gradient of activated serum induced the alignment of neutrophils such that this locomotory morphology was uniform in the observed neutrophil population.

In related studies, cytochalasin B and colchicine were used to explore the role of microfilaments and microtubules in the neutrophil orientation and migration response to activated serum. Cytochalasin B (3.0 μg/ml) prevented migration and decreased the microfilaments seen, but allowed normal orientation of neutrophil structures. In an activated serum gradient, colchicine, but not lumicolchicine, decreased the orientation of nuclei and centrioles, and caused a decrease in centriole-associated microtubules in concentrations as low as 10^{-8} to 10^{-7} M. These colchicine effects were associated with the rounding of cells and impairment

of pseudopod formation. The impaired pseudopod formation was characterized by an inability to form pseudopods in the absence of a solid substrate, formation of narrow pseudopods within a substrate, and a defect in pseudopod orientation in an activated serum gradient. Functional studies of migration showed that colchicine, but not lumicolchicine, minimally decreased activated random migration and markedly inhibited directed migration, but had no effect on random migration. These studies show that, although functioning microfilaments are probably necessary for neutrophil migration, intact microtubules are essential for normal pseudopod formation and orientation, and maximal unidirectional migration during chemotaxis.

Polymorphonuclear leukocytes or neutrophils are capable of ameboid locomotion that can be demonstrated in vitro. In the absence of a chemotactic factor, some neutrophils are at rest and others move without a preferred direction. This nondirected migration has been called random migration. If a chemotactic factor is added in a uniform concentration to the fluid medium, neutrophil random migration is increased; this has been called activated random migration. If neutrophils are incubated in the presence of a gradient of chemotactic factor, the activated random migration becomes directed, and the cells migrate en masse toward the source of chemotactic factor; this is chemotaxis (13, 23).

Observations of leukocytes migrating on solid substrates suggest that in each cell there is a specific internal polarization relative to the direction of migration. The nucleus is at the rear of the cell, the granule-containing cytoplasm lies in front of the nucleus, and a stiff hyaline cytoplasm occupies the pseudopods which extend out from the leading end (1, 18, 19). Although the direction of migration has been postulated to be controlled, in part, by the increased assembly of microtubules associated with a chemotactic stimulus (12), the mechanism for the control and the precise makeup of the internal cell polarization are not known. Moreover, it is not known whether the observed morphologic organization is directly induced by chemotactic factors or is a consequence of migration.

The purpose of the present study was to analyze the effects of gradients of chemotactic factors on the internal structure of human neutrophils. A method was developed for maintaining a population of cells in fixed orientation towards a chemoattractant, using conventional chemotactic chambers with very small (0.45 μm) micropore filters. The small pores permit pseudopod penetration, but cell migration into the fiters is prevented, and, with appropriate stimulation, a monolayer of oriented leukocytes results. This technique is utilized to describe the morphologic processes associated with cell orientation.

In addition, the chemical probes cytochalasin B and colchicine were used to further delineate the possible role of microfilaments and microtubules in orientation and migration. Cytochalasin B inhibited neutrophil locomotion (3, 26), possibly by interfering with a cellular actin-myosin contractile system (14). In some studies, cytochalasin B has also been shown to disrupt microfilaments, leading to the presumption that microfilaments are involved in cellular contractile mechanisms and migration (2, 24).

Colchicine binds to tubulin (8, 25), and in concentrations greater than 10^{-7} M, it has been shown to decrease microtubule assembly in a dose-response fashion in neutrophils (15). The vector of motion during directed migration (chemotaxis) has been attributed to the microtubules which polymerize during interaction of leukocytes with chemotactic factors (12). Much of the speculation that microtubule polymerization is required for directed migration is based on the earlier observation that antitubulins such as colchicine inhibit chemotaxis, but not the speed of locomotion (7, 17, 21).

It will be demonstrated that a gradient of endotoxin-activated serum (conditions of chemotaxis) induces a specific orientation of neutrophil internal structures, and that this orientation rapidly changes in response to changes in direction of the activated serum gradient. Neutrophil migration is not necessary for the initiation or maintenance of this orientation, and may be critical for stabilizing the cell and maintaining the vector of locomotion during chemotaxis.

MATERIALS AND METHODS

Materials were obtained as follows: Hypaque (Winthrop

Laboratories, New York); Ficoll (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.); dextran T-250 (Pharmacia Fine Chemicals); trypan blue dye (Grand Island Biological Co., Grand Island, N. Y.); Gey's tissue culture medium, pH 7.2, containing 2% bovine serum albumin and 2% penicillin and streptomycin (Microbiological Associates, Bethesda, Md.); *Escherichia coli* endotoxin O127:B6 lipopolysaccharide B (Difco Laboratories, Detroit, Mich.); 13 mm 0.45-μm cellulose nitrate micropore filters (Millipore Corp., Bedford, Mass.); 13 mm 3-μm cellulose nitrate filters (Sartorius, Beckman Instruments Inc., Science Essentials Co., Mountainside, N. J.); glutaraldehyde (Ladd Research Industries, Inc., Burlington, Vt.); sodium cacodylate (Fisher Scientific Co., Pittsburgh, Pa.); osmium tetroxide (Polysciences, Inc., Warrington, Pa.); potassium dichromate, uranyl acetate, and propylene oxide (Fisher Scientific); Araldite 6005, Epon 812, and toluidine blue (Ladd Research Industries, Inc.); 51-chromium (^{51}Cr, Amersham/Searle Corp., Arlington Heights, Ill.); latex beads (1.0 μm, Dow Diagnostics, Dow Chemical Co., Indianapolis, Ind.); cytochalasin B (Aldrich Chemical Co., Inc., Milwaukee, Wis.); cholchicine (Sigma Chemical Co., St. Louis, Mo.).

Preparation of Neutrophils

Heparinized whole peripheral blood from normal human donors was separated into a granulocyte-rich fraction by Hypaque-Ficoll and dextran sedimentation techniques (6). This routinely resulted in a cell fraction containing over 95% neutrophils with 95% viability, as determined by exclusion of trypan blue dye.

Neutrophils were suspended in Gey's medium at 2.5 $\times$ 10^6 cells/ml before use. All results represent observations of neutrophils from at least three different donors.

Chemoattractant

Endotoxin-activated serum was prepared as previously described (10) by incubating 30 μg of *E. coli* endotoxin with 0.1 ml of fresh serum in 0.9 ml of veronal buffer, National Institutes of Health Media Unit, for 1 h at 37°C. The reaction was terminated by heating for 30 min at 56°C. The activated serum was diluted 1:1 with Gey's medium, and then used without further treatment.

Preparation of Chemotactic Chambers

Previously described chemotactic chambers were used (11). The upper and lower compartments were separated by a 13-mm 0.45-μm pore size micropore filter. This pore size allowed diffusion of soluble molecules such as chemotactic substances, but prevented cell migration through the filter. Neutrophils were added to the upper compartment (0.8 ml containing 2.5 $\times$ 10^6 neutrophils/ml) and incubated under the following conditions for 45 min at 37°C: (*a*) Buffer in the upper and lower compartments (conditions of random migration). (*b*) Chemoattractant in equal concentrations in both upper and lower compartments (conditions of activated random migration). (*c*) Chemoattractant in the lower compartment only, resulting in a chemical gradient of chemoattractant was then added to the upper compartment, taxis).

In some experiments, the gradient of chemoattractant was reversed for varying periods of time. The reversal of the gradient was done by using chemotactic chambers that had been incubated under conditions of directed migration for 45 min as just described. The buffer was aspirated from the upper compartment, and chemoattractant was removed from the lower compartment. Chemoattractant was then added to the upper compartment, and buffer was added to the lower compartment. This resulted in a chemical gradient of chemoattractant that was opposite in direction to the initial incubation. These chambers were then incubated for 1–30 min at 37°C.

The process was terminated in all chambers by adding cold 2% glutaraldehyde in 0.1 M Na cacodylate, pH 7.3, to the upper compartments after aspiration of the fluid contents of the chambers.

Directed migration (chemotaxis), activated random migration, and random migration were quantitatively assessed by incubating neutrophils in chemotactic chambers in experiments with 3-μm filters run in parallel with experiments with 0.45-μm filters. Both a radioassay (11), which measures the number of ^{51}Cr-labeled cells traversing a top filter and migrating into a lower filter, and a morphologic assay (27), measuring the number of cells migrating varying depths into the filter, were used to evaluate the effect of colchicine and lumicolchicine upon migration.

Cytochalasin B, Colchicine, and Lumicolchicine Treatment

For morphology experiments with cytochalasin B, neutrophils were incubated for 45 min at 37°C under conditions of directed migration in chemotactic chambers using 0.45-μm pore filters. Media were aspirated and replaced with media containing cytochalasin B (3 μg/ml), either maintaining the original direction of the gradient of chemoattractant, or reversing the direction of the gradient as previously described. After incubation for 30 min at 37°C, the cells were fixed and the filters were embedded for sectioning. Control chambers were processed in the same way, using media without cytochalasin B.

For studies with colchicine, neutrophils were incubated for 30 min at 37° in suspension in either buffer only or buffer containing the indicated concentrations of colchicine, and then placed in either chemotactic chambers containing 3.0-μm filters for functional studies of locomotion, or in chambers with 0.45-μm filters under conditions of directed migration for morphology studies. In the morphology experiments, after 45 min of incubation, the gradient of chemoattractant was either maintained or reversed for 5–30 min as previously described.

Additional controls for the colchicine experiments were run, using lumicolchicine prepared by UV irradiation of colchicine as previously reported (22).

Fixation and Embedding of Filters with Adherent Cells

Filters were removed from the chambers and placed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, on ice for 30 min. After a rinse in sodium cacodylate buffer, the filters were postfixed in Dalton's chrome osmium (1%) buffer for 30 min. The filters were stained with 0.5% aqueous uranyl acetate for 40 min and then rapidly passed through a graded series of ethanol. After two washes in 100% ethanol, propylene oxide was added to the final ethanol wash, a few drops at a time, with gentle agitation until the filter began to become translucent. Keeping the volume small and slowly adding the propylene oxide prevented the filters from curling up into tight cylinders. The ethanol-propylene oxide mixture was removed, and two washes of 100% propylene oxide were added. The filter completely cleared so that only the layer of cells was seen. The filter with cells was fragile and could not be touched, but it could be transferred by gently pouring into a shallow, round, embedding mold. In this way, the orientation of the cells was maintained.

The propylene oxide was aspirated by a Pasteur pipette and a 1:1 mixture of Epon-Araldite and propylene oxide was added. After 10 min this was removed and 100% Epon-Araldite was added. The molds were allowed to sit overnight in order to evaporate residual propylene oxide, and then were cured at 80°C for 24 h.

In this way, the entire filter was embedded in a known orientation. Thick sections were cut with glass knives perpendicular to the filter surface across the entire diameter of the filter. They were stained with toluidine blue, and areas were selected for thin sections and electron microscopy. Thin sections were cut on a LKB ultramicrotome III (LKB Instruments, Rockville, Md.) with a diamond knife, stained with uranyl acetate and lead citrate, and examined on a Hitachi 11E or Philips 300 electron microscope at 75 kV and 80 kV accelerating voltage, respectively.

Quantitation of Polarization of Cellular Internal Structures

The vertical location of the nuclei of neutrophils with respect to the filter surface was determined by examining light micrographs of toluidine blue-stained, thick sections of filters. This axis is parallel to the chemoattractant gradient under conditions of directed migration. Measurements were made of both the vertical distance from the portion of the neutrophil surface farthest from the filter surface, or top of the cell, to the nearest lobe of the nucleus (distance T), and the distance from the lowest lobe of the nucleus to the part of the cell pseudopods extending into the filter or the bottom of the cell (distance B). The fraction B/B + T expresses the vertical position of the nucleus within the cell, where the whole vertical cell length is 1.0. The bottom of the cell is 0, the mid-point is 0.5, and the top of the cell is 1.0. Because the units of measurement cancel out in the calculation, the measurements from cells of various shapes and magnifications can be determined and subjected to statistical analysis (Student's t test).

The position of the centriole was determined by examining serial sections of 12–20 adjacent cells in the electron microscope. Although a centriole could be seen only in one of 10–20 random cell sections, serial sectioning allowed careful analysis of the centriole position in 35–80% of cells examined. In this way, the vertical position of the centriole with respect to the cell nucleus was determined. The centriole was classified as located above or below the nucleus. If the centriole was to the side of the nucleus or occupied a position between several lobes, the position was classified as middle. The position of the centriole was given a weighted value of +1 (above the nucleus), 0 (middle), or −1 (below the nucleus). A mean score was determined by adding the weighted values and dividing by the total number of located centrioles. This mean score was tested for significance from 0, the expected value for random distribution of centrioles, and was also subjected to chi-square analysis for correlation of the mean score with direction of the gradient of chemoattractant.

Microtubule Counts

Quantitation of centriole-associated microtubules was determined by locating cells in which some portion of the centriole had been cut in the plane of that cell section. Examination of serial sections was specifically avoided so as not to prejudice selection of those sections of the same centriole associated with more or less microtubules. Adjacent cells on the filter were each examined at high magnification to determine whether or not a centriole was present in that section. When a centriole was located, all the microtubules were counted in a 1.0-μm radius from the centriole. Microtubules were defined as parallel-walled structures 250 Å across, and at least 100 nm in length. Microtubules cut in cross section, appearing as a 250 Å diameter circle with a radiolucent area surrounding it, could be identified, but were not counted in order to avoid confusion with other cellular structures. Counts from at least 8–14 cells were done for every condition examined, and subjected to statistical analysis (Student's t test).

RESULTS

Cell Orientation During Directed Migration

Using both the ^{51}Cr radioassay of chemotaxis (11), and the morphologic assay of Zigmond and

Hirsh (27), we first verified that the activated sera used for these experiments was capable of activating random migration and, under appropriate conditions, of converting random migration to directed migration (chemotaxis).

Using the same preparations of activated serum and neutrophils under conditions of random, activated random, and directed migration, the 0.45-μm mesh filters did not allow migration of any cells into the filters after a 45-min incubation. However, there are clear differences in the morphology of the neutrophils at the surface of the filter after incubation under these three conditions of migration. Fig. 1 is a composite of low-magnification electron micrographs of neutrophils on the surface of 0.45-μm pore size filters.

Under conditions of random migration (Fig. 1*a*), some neutrophils are attached to the filter but do not show evidence of polarization of internal structure. The nucleus tends to occupy a position in the middle of the cell, and there is almost no pseudopod penetration into the filter. Granules are distributed at random around the nucleus.

Under conditions of activated random migration with a uniform concentration of stimulus, more neutrophils were found at the filter surface (Fig. 1*b*). The neutrophils have a more irregular shape, with evidence of increased pseudopod formation in random directions compared with that seen under conditions of random migration. There is also increased asymmetry in the distribution of the internal contents of the cell, reminiscent of the locomotory morphology that others have previously described at the light microscope level (1). Thus, there is morphologic evidence consistent with an increase in migratory activity induced by the presence of a chemoattractant such as activated serum. However, there is no suggestion of uniform polarization of the cell population.

In the presence of a gradient of chemoattractant (activated serum beneath the filter), there is marked polarization of neutrophil morphology, with all the cells at the surface of the filter oriented in the same direction (Fig. 1*c*). This orientation occurred despite the inability of cells to migrate into the filter, except for some pseudopod penetration. Pseudopods extended only into the filter beneath the cells, and the rest of the cell surface was relatively smooth. The nucleus was displaced above the middle of the cell, and the bulk of the cytoplasm containing most of the granules was beneath the nucleus.

The average location of nuclei in the vertical axis of the neutrophils was determined, as outlined in Methods, during each of the three conditions of migration (Fig. 2). In random migration, the average position of the nucleus was 0.49 ± 0.04. This did not significantly differ from 0.5 ($P > 0.20$). Conditions of activated random migration resulted in an average position of 0.44 ± 0.06 and also did not differ significantly from 0.5 ($P > 0.20$). With directed migration there was a significant displacement of nuclei from midcell position to the side of the cell away from the source of the chemoattractant (average nucleus position of 0.72 ± 0.02; $P < 0.001$).

Effect of Changing Location of Chemoattractant on Cell Orientation

Chemotactic chambers were incubated for 45 min under conditions of directed migration with the source of the gradient of chemoattractant beneath the filter. The gradient was then reversed by placing the activated serum on top of the cells as outlined in Methods. This resulted in a rapid reorientation of cells in a direction opposite to the initial polarization (Fig. 3). 1 min after reversal, blunting of pseudopods was noted and the nuclei occupied a lower position within the cell (Fig. 3*a*). In many cells, nuclei actually entered the retracting pseudopods that were still within the filter; nuclei were rarely seen within the pores of the filter when the stimulus was beneath the filter before reversal. It is important to note that pseudopods did not immediately form at the upper surface of the cells.

5 min after reversal of the gradient (Fig. 3*b*), pseudopod retraction was complete and the nuclei were located toward the lower end of the cells, away from the source of the chemoattractant. At this time, the leading end of the cells, toward the chemoattractant, had broad, veil-like pseudopods extending upward into the free fluid medium. The exact timing of this sequence of events was variable with different neutrophil preparations and different sources of activated serum. However, the repolarization was always complete by 10 min and after longer periods of incubation, progressive cell loss from the surface of the filter was noted. In related studies (not shown), when a 0.45-μm filter was placed on top of the cells concomitant with the change in location of the chemotactic factor, cells actually migrated off the lower filter, and imbedded their pseudopods in the upper filter towards the new direction of the chemoattractant. Thus,

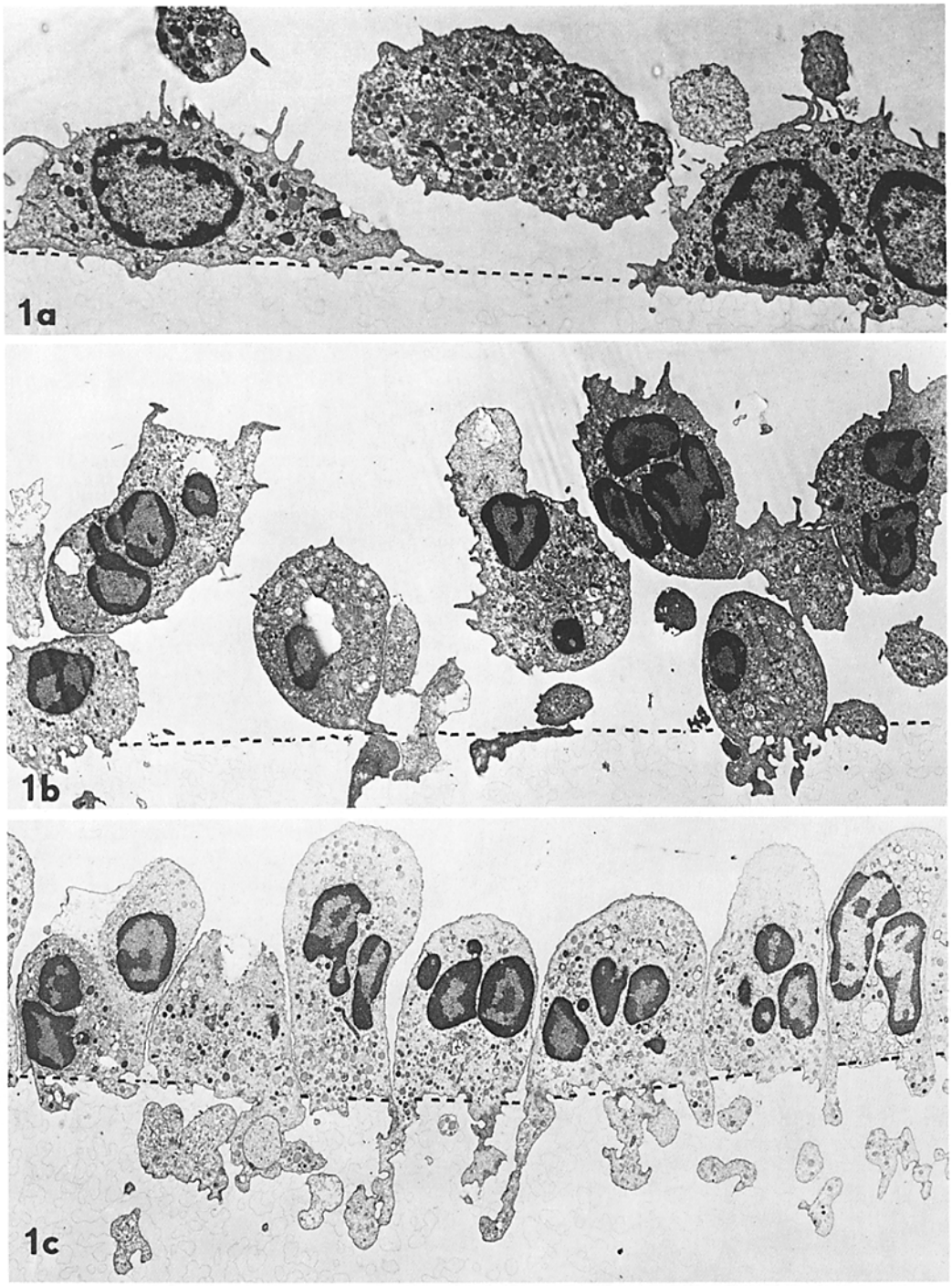

FIGURE 1 Composite electron micrograph of neutrophils at the surface of 0.45-μm pore filters after 45-min incubation under conditions of random (*a*), activated random (*b*), or directed migration (*c*). The filter surface is indicated by the dashed line. No chemotactic factor is present in (*a*); chemotactic factor is present on both sides of the filter in equal concentrations (*b*); and in (*c*) chemotactic factor is present only below the filter. See text for details. (*a*) × 6,800, (*b*) × 3,300, (*c*) × 3,450.

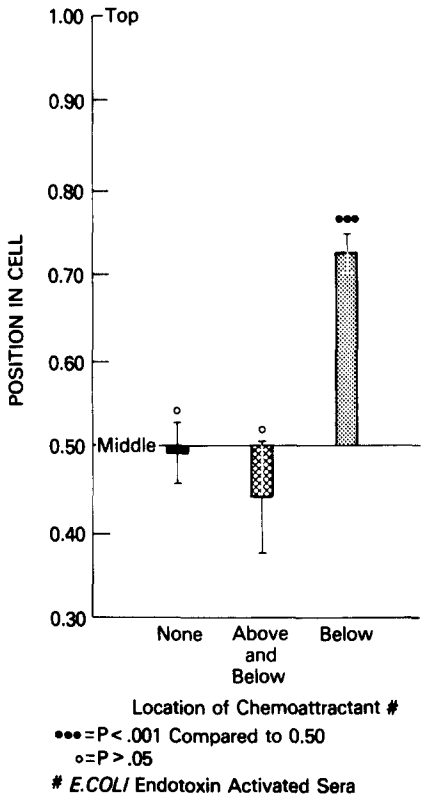

FIGURE 2 Average position of the nucleus in the vertical axis of neutrophils incubated under conditions of random migration (left bar), activated random migration (middle bar), and directed migration (right bar), corresponding to the neutrophils depicted in Fig. 3. The ordinate represents the relative position of the nucleus within a cell of unit height 1.00. The bottom of the cell nearest the filter is 0 and the middle of the cell 0.50. The average position of the nucleus was determined as described in Methods, and plotted as a function of the deviation from the middle of the cell. The bars depict the mean ± SEM of 30 determinations.

the loss of cells from the filter with prolonged incubation represented a change in direction of migration, and not merely a decrease in cell adherence to the lower filter.

The average position of the nucleus at 1 and 5 min after reversal of the direction of migration was determined as previously described, and shown in Fig. 4. 1 min after reversal, the nuclei were significantly ($P < 0.01$) away from the center of the cell toward the upper side, although significantly different from their prereversal location (0.72 ± 0.02 vs. 0.58 ± 0.03, $P < 0.05$). 5 min after reversal of the chemoattractant gradient, the average position of the nucleus had become 0.39 ± 0.03, significantly below midcell position ($P < 0.001$). Thus, a rapid and significant reorientation of the nucleus, in response to a change in the direction of the chemoattractant gradient, preceded the migration of cells toward the new source of the chemoattractant.

Location of Submembranous Microfilaments During Conditions of Chemotaxis

Fig. 5*a* shows a neutrophil during directed migration, with the source of the chemoattractant below the filter. This cell demonstrates the characteristic orientation of the nucleus toward the top of the cell. Most of the granule-containing cytoplasm is located below the nucleus and in the pseudopods which extended into the filter pores. The matrix of the filter stains as thin electron-dense lines. The cell is relatively smooth at the sides and top without evidence of pseudopod formation, except beneath the cell.

Just inside the cell and directly next to the plasma membrane on all sides of the cell are microfilaments which appear as irregular, fuzzy collections of varying electron density (labeled *mf*), at the magnification of Fig. 5*a*. Microfilaments at the sides and top of the cell occur as a narrow irregular layer. At the lower end of the cell and in the pseudopods, the microfilament collections occupy a greater area extending farther from the plasma membrane. In addition, at the lower end of the cell there are larger, discrete, very dense condensations of microfilaments which are shown by arrows in Fig. 5*a* and at higher magnification in Fig. 5*b*. At the higher magnification, the microfilaments appear as a fine meshwork of filaments ~ 56–60 Å in diameter. The densest condensations are restricted to areas where the plasma membrane closely approaches the filter matrix and may represent sites of adhesion (arrows, Fig. 5*b*).

Orientation of Centrioles and Associated Microtubules During Conditions of Chemotaxis

We have previously shown that human neutrophils exposed to chemotactic factors have an increased assembly of centriole-associated microtubules, and it was hypothesized that oriented assembly of microtubules is important in providing the vector of motion during chemotaxis (12). In the current experiments, the centriole is the focus

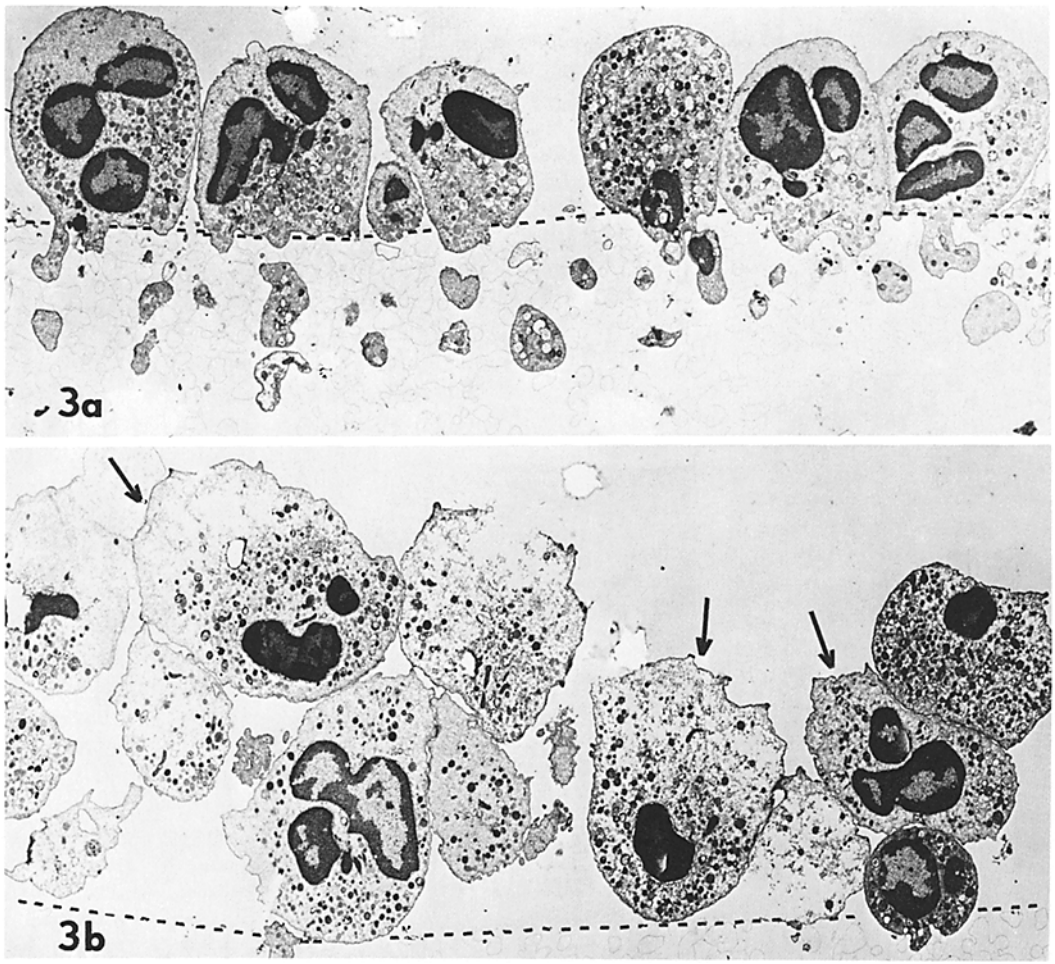

FIGURE 3 Electron micrographs showing the effect of reversal of the direction of the gradient of chemoattractant at 1 min (*a*) and 5 min (*b*). These cells were initially oriented as shown in Fig. 1*c*, and then the gradient was reversed as described in Methods. The filter surface is indicated by the dashed line. Note that the nuclei occupy a lower position in many cells even after 1 min (*a*), and the upper surface of the cells does not yet show evidence of pseudopod formation. By 5 min (*b*) the cells have withdrawn from the filters, nuclei have oriented toward the bottom of the cell, and pseudopods extend upward toward the chemoattractant source (see arrows). (*a*) × 3,600, (*b*) × 3,300.

of a radial array of microtubules (*mt*) which attach to electron-dense areas (*d*) near the centrioles (Fig. 5*c*). Serial sections demonstrated that most, if not all, microtubules in the neutrophil originate at the centriole and radiate out to all parts of the cell into, but not through, the periphery occupied by submembranous microfilaments. It is of note that the microtubules extend both upward between the nucleus lobes and downward into the pseudopods. The Golgi apparatus of the neutrophil (Fig. 5*a*) was usually found near the centriole.

Effect of Changing Location of Chemoattractant on the Position of Centrioles, Microtubules, and Microfilaments

As noted (Figs. 3, 4), a reversal of direction of the gradient of chemoattractant resulted in a rapid reorientation of the nucleus. High magnification electron micrographs of the early events after reversal of the gradient reveal a complex series of morphological events which are shown in Figs. 6–8.

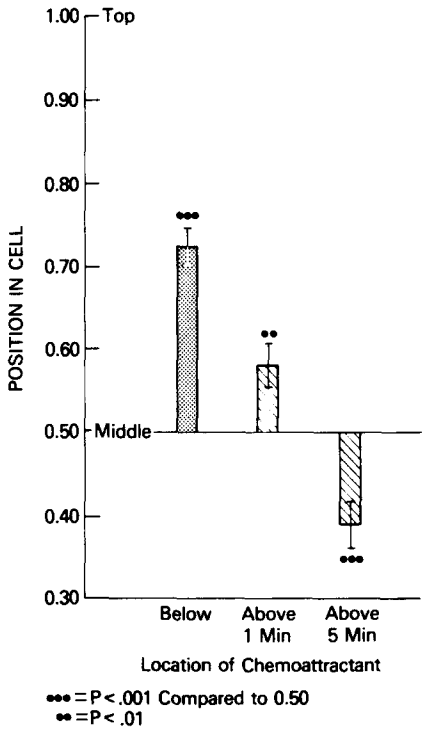

FIGURE 4 Average position of the nucleus after reversal of the direction of the gradient of chemoattractant for 1 min (middle bar) and 5 min (right bar). The left bar shows the average position of the nucleus under conditions of directed migration before reversal of the gradient. The graph is constructed as described for Fig. 2, and bars and brackets represent the mean ± SEM, 30 determinations.

The earliest response, within 1 min of reversal of the chemoattractant gradient, is a paradoxical movement of the centriole down and away from the nucleus, and away from the new location of the chemoattractant. This can be seen in Fig. 6.

Fig. 7, a higher magnification in another cell, shows that this initial response may carry the centriole deep into the pseudopod within the filter pores. The microtubule array continues to be associated with the centriole in its new position. At the same time, increased dense bundles of microfilaments can be seen preceding pseudopod retraction.

After this initial movement of the centriole into the pseudopods, several other events occur. The pseudopods appear to retract while the nucleus drops to the lower end of the cell away from the chemoattractant. In some cells, the nucleus actually enters a retracting pseudopod within the filter (Fig. 3*a*). Moderately dense condensations of microfilaments are sometimes seen beneath the plasma membrane on the upper side of the cell, towards the chemoattractant. In other cells (not shown), the nucleus and centriole lie in a plane parallel to the filter surface as the centriole reorients by movement around the nucleus. 5–10 min after reversal of the chemotactic gradient, the centriole occupies a position above the nucleus, while the nucleus has completed reorientation to the bottom of the cell away from the chemoattractant (Fig. 8). The microtubules radiating from the centriole region have also reoriented to the new leading side of the cell. At the leading end of the cell, closest to the chemoattractant, a broad, veil-like pseudopod containing a loose network of microfilaments is noted. Note that in the absence of sites of adhesion to the filter, the increases in microfilaments in the leading pseudopods do not form focal, dense condensations seen at the bottom of the cell in Fig. 5*a*.

The position of the centriole was determined by examining 12–20 adjacent cells by serial sectioning (see Methods). Centriole orientation in the three conditions of migration and after reversal of the chemoattractant gradient was subjected to statistical analysis (Table I). Under conditions of random and activated random migration, there was no preferred orientation of the centriole. In contrast, as shown by the representative cell in Fig. 5*a*, under conditions of directed migration, a definite orientation of the centriole within the cell to the side of the nucleus toward the chemoattractant source was noted. The χ^2 analysis of the correlation of direction of chemoattract source above or below the cells with position of centriole above or below the nucleus was highly significant ($P < 0.001$).

Thick Filaments

In addition to microtubules and microfilaments, a third filamentous structure can be seen in neutrophils. These filaments, ~ 100 Å in diameter, have been seen in a number of cell types (5). As shown in Figs. 5*a* and 7, these filaments, labeled F in the figures, are easily differentiated from microfilaments because of their larger diameter, because they occur as discrete, long, individual filaments, and because they occupy the central portions of the cell body and the core of the pseudopods rather than the periphery. With reversal of the direction of the gradient of chemoattractant, these filaments appear to realign in a configuration that often parallels changes in microtubule

orientation. The function and chemical structure of these filaments are not known.

Effect of Cytochalasin B on Neutrophil Orientation under Conditions of Directed Migration

In these experiments, and others, it has been shown that cytochalasin B completely inhibits neutrophil migration (3, 12, 26).

The effects of incubating neutrophils with cytochalasin B on cell orientation can be seen in Fig. 9*a*, *b*, which is a composite low magnification electron micrograph. In Fig. 9*a*, in which the gradient of chemoattractant was maintained with the chemotactic source beneath the filter, the cells remained oriented on the filter with the overall cell shape resembling untreated cells. Extensive loss of pseudopods penetrating the filter and some cellular blebbing (arrows), characteristic of cytochalasin B-treated cells (4), were noted (Fig. 9*a*), and the nuclear lobes tended to be less tightly grouped together than in control cells. When the gradient of chemoattractant was reversed for 30 min in the presence of cytochalasin B (Fig. 9*b*), the neutrophils did not migrate off the filter, as occurred in control media without cytochalasin B (Fig. 3*b*). Despite these effects of cytochalasin B, the nuclei still significantly oriented in the cell away from the chemoattractant source (Fig. 10). With the chemoattractant beneath the filter, the cytochalasin B-treated neutrophils had an average nuclear position of 0.61 ± 0.02 ($P < 0.001$, compared to mid-cell position of 0.5). Although the orientation of the nuclei in cytochalasin B-treated cells (quantitated in Fig. 10) was significantly different from that of control cells (0.72 ± 0.02 vs. 0.61 ± 0.02, $P < 0.05$), this difference was due to the lack of deep penetration of pseudopods in cytochalasin B-treated cells. The decreased pseudopod penetration diminished the distance from the leading edge of the cell to the filter surface. When the nuclear position of cytochalasin B-treated cells and control cells were compared by measuring the position from the filter surface, no significant difference was noted. Furthermore, after reversal of the chemotactic gradient, the nuclei of cytochalasin B-treated cells were significantly reoriented to the bottom of the cell as seen in Figs. 9*b* and 10 (average nuclear position of 0.38 ± .02, $P < 0.001$, compared to midcell position of 0.5).

Fig. 11 is a higher magnification of a neutrophil treated with cytochalasin B, with the chemoattractant gradient source below the cell and filter (corresponding to the conditions seen in Fig. 9*a*).

After cytochalasin B treatment, there is a marked decrease in the amount of microfilaments beneath the plasma membrane, although some discrete, electron dense condensations (labeled *mf*) can be noted in Fig. 11. The almost continuous, thin layer of filaments seen at the sides and top of the untreated oriented cell in Fig. 5*a* is lacking in the cytochalasin B-treated cell in Fig. 11. Instead, there are a few discrete, irregular densities. At the same time, the numerous, large, dense condensations of microfilaments, usually seen at what are probably sites of adhesion to the filter in the untreated oriented cell in Fig. 5*a* and *b*, are markedly diminished in area and number in the cytochalasin B-treated cell in Fig. 11.

We have shown that there is a high degree of correlation between the location of the centriole and its associated array of microtubules, and the direction of a gradient of chemoattractant. As can be seen in Fig. 11, the centriole normally orients to a position in the cell to the side of the nucleus nearest the chemoattractant source, despite the inhibition of migration by cytochalasin B. As shown in Table II, serial section examination of adjacent cells showed this centriole orientation to be highly significant.

Microtubule assembly was unaffected by cytochalasin B treatment. Serial sectioning demonstrated that they extended radially from the centriole to all parts of the neutrophil as in untreated cells. However, a subtle difference was that in untreated cells the microtubules often followed a gently curving course, particularly near the cell periphery, whereas in the cytochalasin B-treated cells, the microtubules radiated out in almost straight lines from the centriole.

Effect of Colchicine on Neutrophil Structure

Control cells, incubated in buffer before being subjected to the conditions of directed migration, oriented normally on the filter surface. However, cells incubated and maintained in 10^{-5} M colchicine did not orient normally in a gradient of chemotactic factor (Fig. 12). The most striking effect of colchicine was the rounded appearance of the cells and the lack of normal nuclear orientation. Measurements of nuclear location in these cells showed no correlation with the direction of

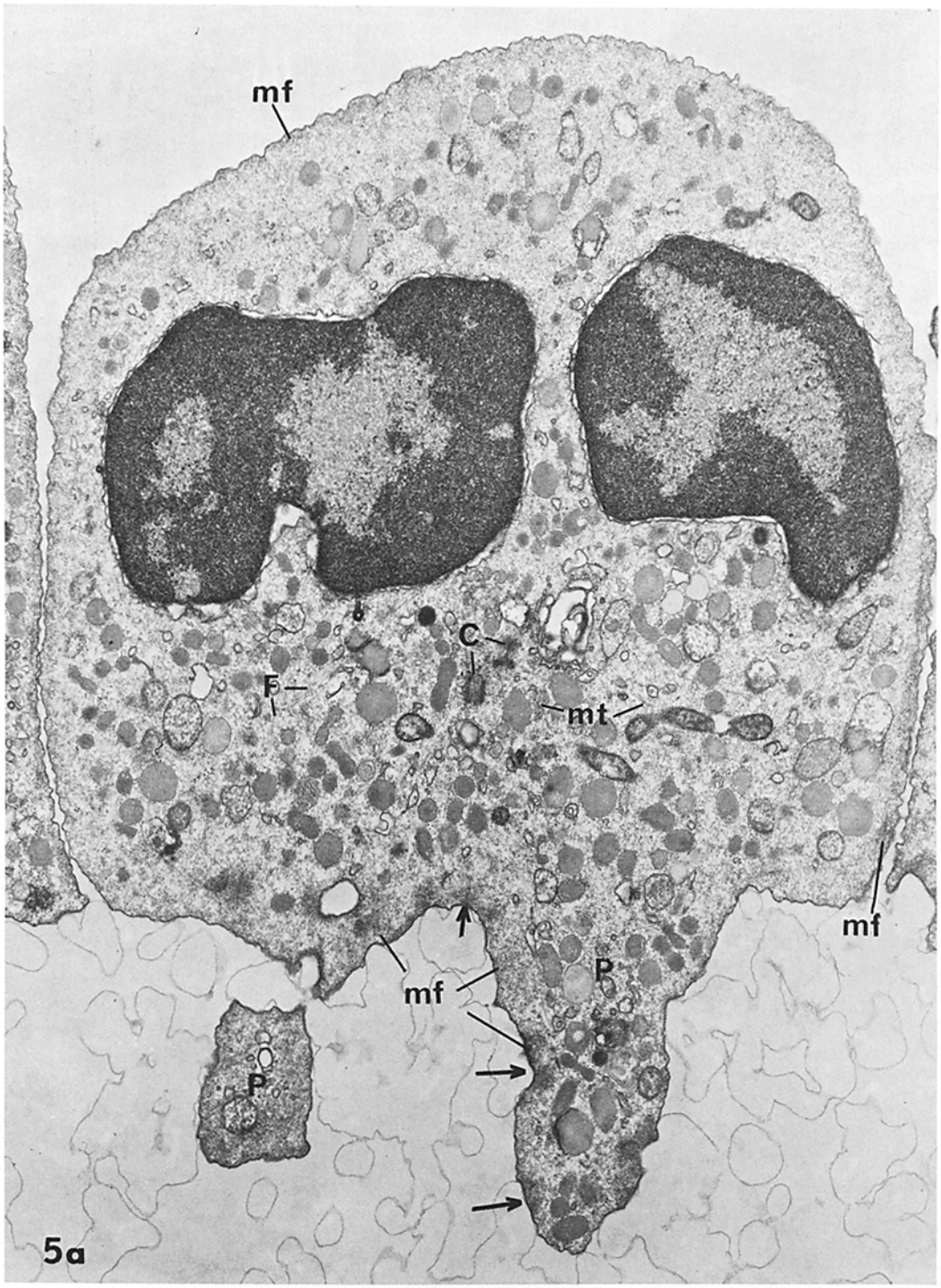

FIGURE 5*a* High magnification electron micrograph of a neutrophil oriented in a gradient of activated serum with the source of 5% activated serum beneath a 0.45-μm pore filter. The nuclear lobes are oriented toward the upper part of the cell, and the bulk of the granule-containing cytoplasm lies beneath the nucleus. A thin layer of submembranous microfilaments (*mf*) can be seen at the sides and top of the cell, and at the lower end and in the pseudopods (*P*) is a thicker layer of submembranous microfilaments (*mf*), in which loose aggregations of microfilaments alternate with focal areas of increased density (arrows). The centriole is indicated by *C*, bundles of 100 Å thick filaments are indicated by *F*, and microtubules are indicated by *mt*. × 15,340.

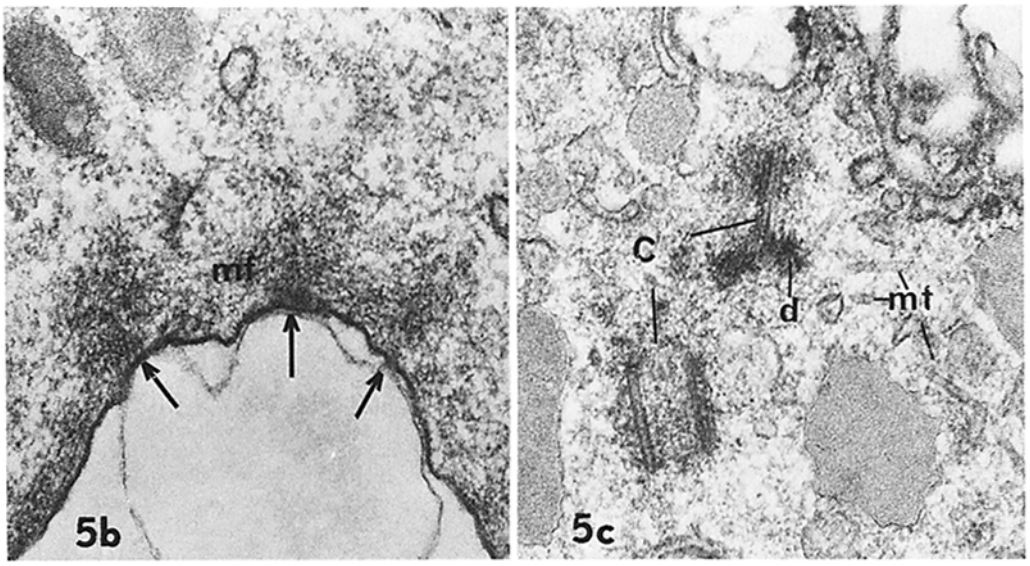

FIGURE 5*b, c* Submembranous microfilaments (*mf*) at the lower end of the neutrophil shown in Fig. 5*a*. The microfilaments consist of a polygonal meshwork of 50–60 Å filaments. Local dense concentrations of filaments occur at sites of close contact, possible adhesion with the filter matrix (arrows). × 58,500. Higher magnification of the centriole (*C*) of the neutrophil shown in Fig. 5*a*. Microtubules (*mt*) appear to radiate from dense areas (*d*) associated with the centriole. × 58,500.

chemoattractant gradient, and the average nuclear position did not differ from 0.5 or midcell position (Fig. 13). In addition, examination of serial sections of adjacent cells indicated that there was no correlation between the centriole position and the direction of the gradient of chemoattractant (see Table II). In the cell shown in Fig. 12, the centriole is at the side of the nucleus; no microtubules are associated with this centriole. Microtubules were markedly decreased in number in cells treated with 10^{-5} M colchicine compared to untreated cells, as will be quantitatively shown. However, an increase of 100 Å filaments (F), particularly near the centriole, was noted (Fig. 12). An increase of filaments of this size has been reported in other cells after colchicine treatment (5), but the significance of this finding and the relationship between these filaments and those of similar diameter present in untreated cells is not known.

Colchicine also affected pseudopod formation. As seen in Fig. 12, some of the rounded, colchicine-treated cells responded to a chemoattractant gradient by sending a slender pseudopod into the filter pores. In some respects, the pseudopods of colchicine-treated cells resembled those of control cells. Increases in both the loose meshwork and focal areas of dense microfilaments were observed in the pseudopods. However, unlike those of control cells, the pseudopods were unusually narrow. Many of these narrow pseudopods extended off to the side of the cell within the filter pores and occasionally reemerged from the filter pores (see Fig. 16*c*), suggesting impairment of sustained orientation by the extending pseudopod in response to the chemoattractant gradient.

After 5 min of reversal of the direction of the gradient of chemoattractant, colchicine-treated cells only partially withdrew their extended pseudopods, resulting in shorter and wider pseudopods with dense bundles of microfilaments (not shown). Although the upper cell surface was somewhat more irregular in outline, no pseudopod formation was seen in the new direction of chemoattractant, and cells did not migrate off the filter surface even after a 30-min incubation in the reversed gradient. Submembranous microfilaments were occasionally increased at the upper side of the cell toward the chemoattractant.

It is possible that the filter surface itself may be modulating pseudopod formation and orientation in the colchicine-treated cells. In experiments not shown, this possibility was evaluated. Neutrophils that had been incubated for 30 min in suspension with 10^{-5} M colchicine or in buffer were suspended for an additional 15 min in 5% activated

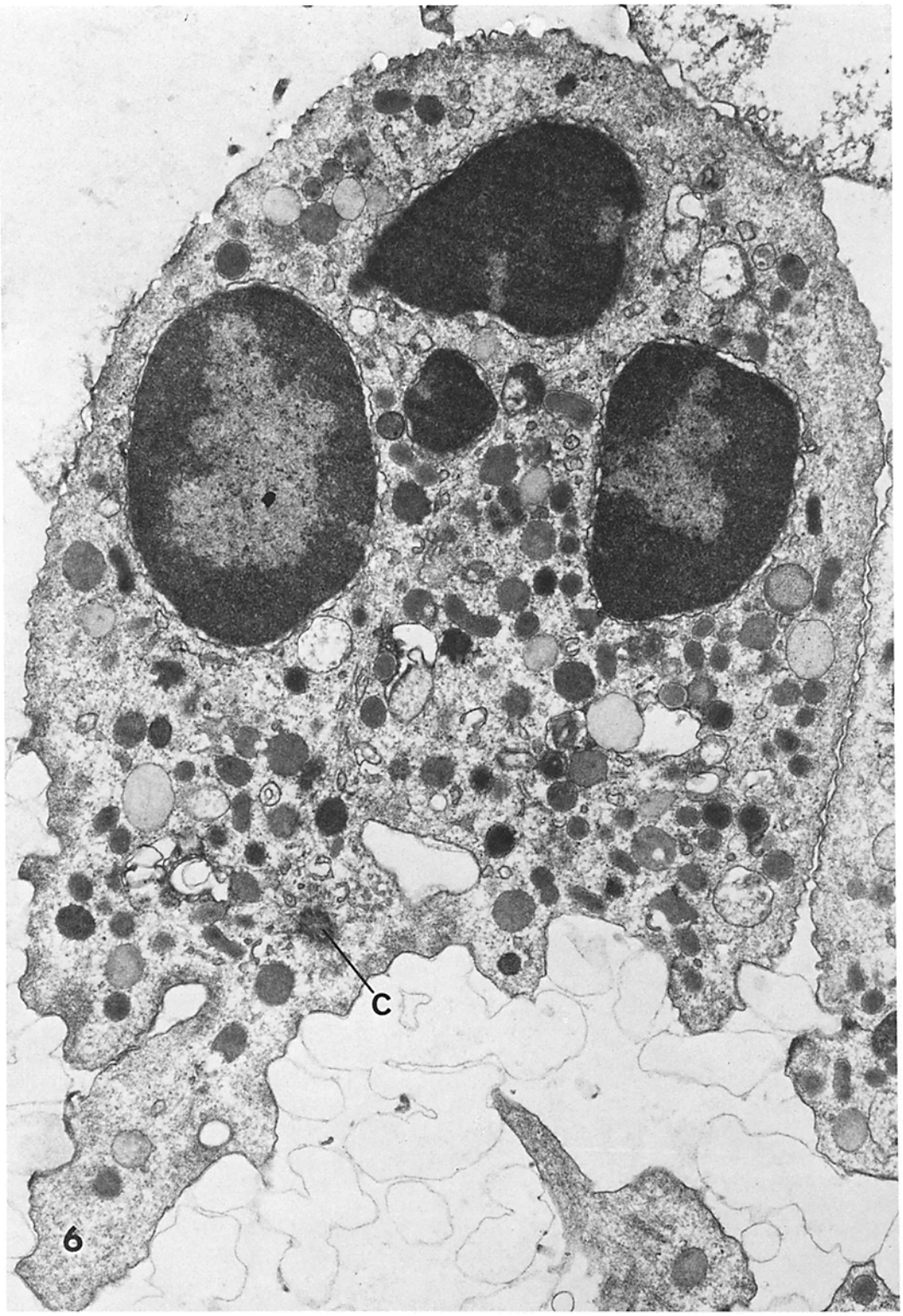

FIGURE 6 1 min after reversal of the direction of the gradient of chemoattractant, the earliest change seen in the neutrophil consists of movement of the centriole (*C*) down and away from the nucleus Compare the position of this centriole with the position of the centriole seen in Fig. 5*a*. × 20,000.

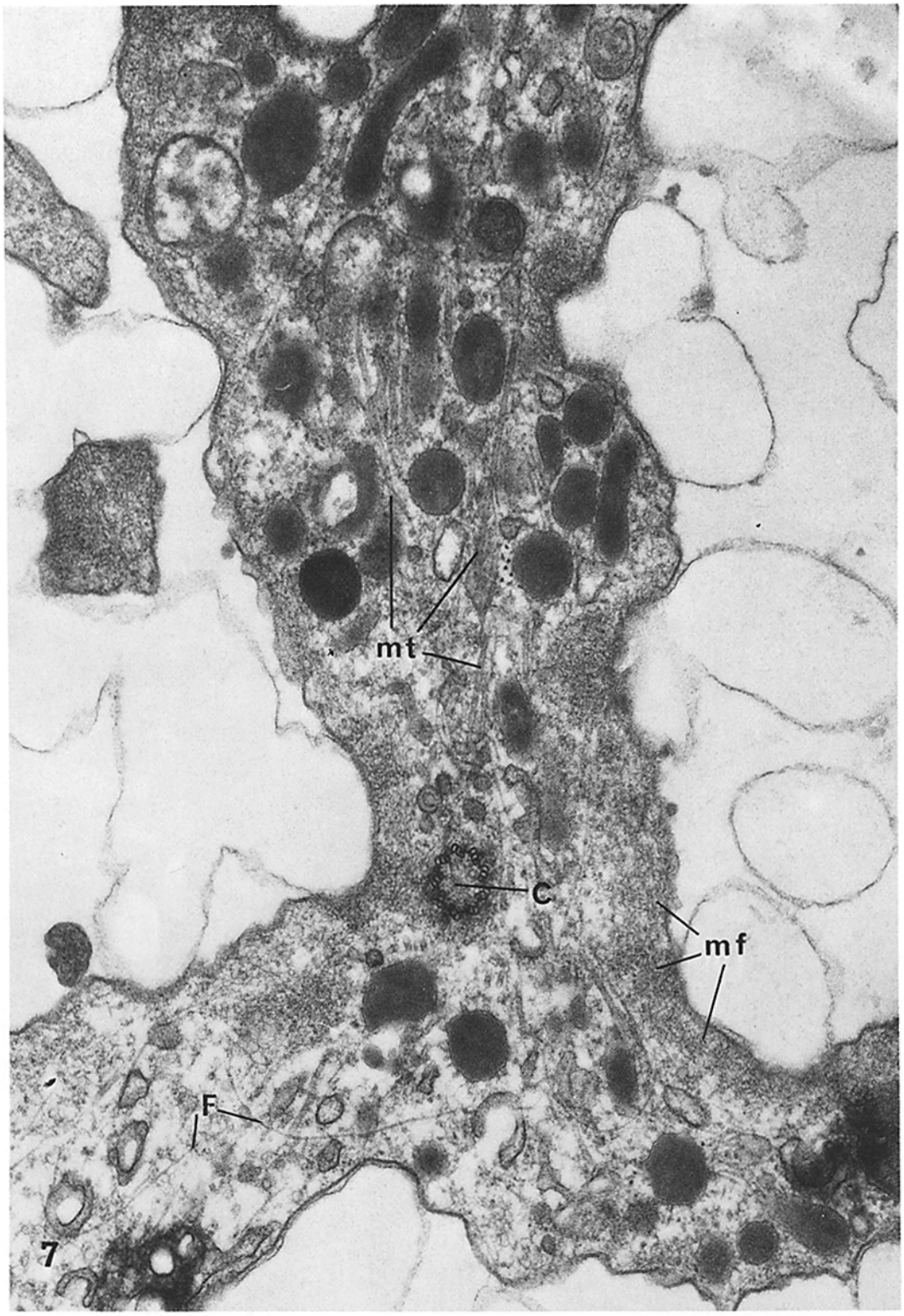

FIGURE 7 Very high magnification (× 48,000) of a neutrophil pseudopod within the pores of the filter matrix, 1 min after reversal of the direction of the gradient of chemoattractant. The centriole (*C*) has migrated deep into this pseudopod. Microtubules (*mt*) radiate out from the centriole both upward into the cell body and down into the periphery of the pseudopod. There are large bundles of submembranous microfilaments (*mf*). Also seen are 100 Å thick filaments (*F*) which in many areas appear to run parallel to the microtubules within the core of the pseudopod.

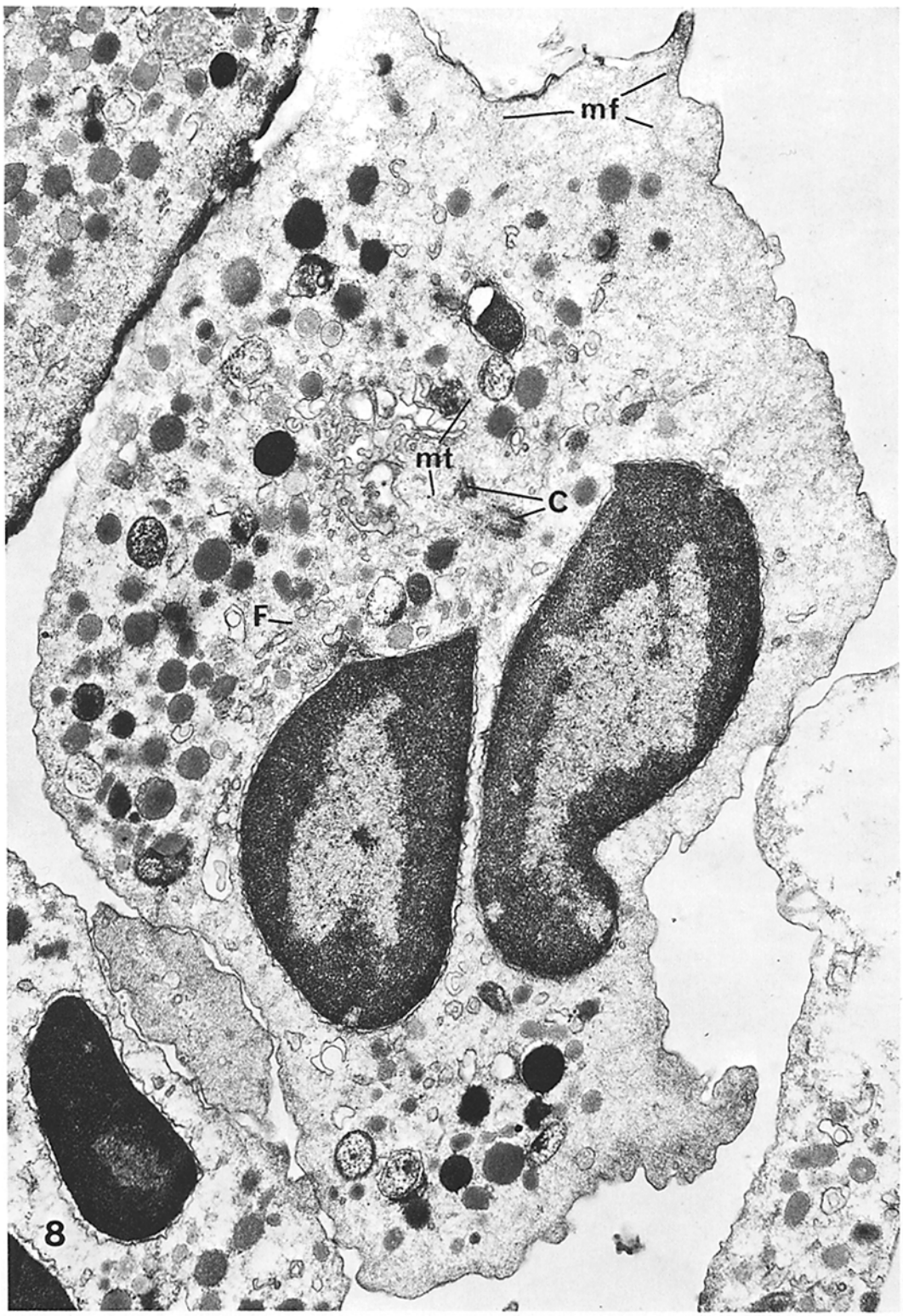

FIGURE 8 5 min after reversal of the direction of the gradient of chemoattractant, this neutrophil has completely reversed orientation and has migrated up between other cells completely off the filter. The nucleus is at the bottom of the cell, and the centriole (*C*) with its radial array of microtubules (*mt*) is located above the nucleus. An increased loose meshwork of submembranous microfilaments (*mf*) occupies the leading end of the pseudopod at the top of the cell. Thick filaments (*F*) of 100 Å diameter are also seen in this cell. × 18,300.

TABLE I

Neutrophil Centriole Orientation During Random, Activated Random, and Directed Migration

Conditions of migration	Number of adjacent cells examined*	Number of cells in which the centriole was located	Position of centriole relative to nucleus (no. at each location) above	middle	below	Mean score‡ ±SD	P§	Correlation of direction of gradient and mean score χ^2	P, χ^2
Random	20	7	3	3	1	+0.29 ± 0.26	>0.2	–	–
Activated random	20	9	5	2	2	+0.33 ± 0.27	>0.2		
Directed (chemotaxis) Chemoattractant beneath cells	12	10	0	1	9	−0.90 ± 0.10	<0.001	21.0	<0.001
Directed (chemotaxis) Chemoattractant above the cells (5 min reverse)	20	12	12	0	0	+1.00 ± 0.00	<0.001		

* 12–20 serial sections extending through 1–1.5-μm depth were examined, using both photographs and freehand drawings to positively identify cells from one section to the next. Centriole position was determined in the vertical cells axis relative to the nucleus position. See Methods.

‡ Mean score $= \frac{\text{number of centrioles above nucleus} - \text{number of centrioles below nucleus}}{\text{total number of centrioles}}$

§ Significance level of difference between mean and 0, calculated from Z = mean score/SD, which was assumed to have a standard normal distribution.

serum or in buffer, then fixed with glutaraldehyde, pelleted, embedded, and sectioned. Cells in buffer, whether colchicine treated or not, were round and had a smooth surface. In the presence of activated serum, the control cells became very irregular in shape, with most cells showing evidence of pseudopod formation as previously reported by Gallin and Rosenthal (12). A suspension of colchicine-treated neutrophils exposed to activated serum showed only slight irregularity of the cell surface and no pseudopod formation. However, when colchicine-treated cells were allowed to settle on a 0.45-μm pore filter in the presence of a uniform concentration of activated serum (conditions of activated random migration), pseudopods extended from the rounded cells into and across the filter surface, but not upward into the liquid medium. Thus, it appeared that colchicine-treated neutrophils had an impaired ability to form pseudopods in suspension, but that the presence of a solid substrate allowed adhesion and pseudopod formation to occur. However, as previously shown, these pseudopods differ from normal in their shape and in their ability to orient in response to a chemoattractant gradient.

Effects of Colchicine on Neutrophil Migration

Because the colchicine-induced impairment of structural orientation in a chemotactic gradient appeared to be associated with an impairment of pseudopod formation and orientation, the effect of colchicine upon neutrophil migration was measured.

Neutrophils were incubated in suspension for 30 min with 10^{-5} M colchicine or in buffer alone. Cells were placed in chemotactic chambers containing 3-μm pore size filters, and incubated for 1 h under conditions of random migration (buffer alone), activated random migration (activated serum in uniform concentration above and below the filter), or directed migration (a gradient of activated serum with the source below the filter). Colchicine (10^{-5} M) was maintained in those chambers containing cells that had been initially treated with colchicine. The filters were stained and cleared, and the number of cells that migrated various distances into the filter was determined (Fig. 14). Colchicine did not affect the ability of neutrophils to undergo random migration. Colchicine caused a small, yet consistent impairment of activated random migration, as demonstrated in Fig. 14 by the shift of the curve of migrating cells to the left. Colchicine dramatically altered the distribution of cells in the filters after incubation under conditions of chemotaxis. The normal en masse migration of cells into the filter was disturbed. The population of colchicine-treated cells in the gradient of chemoattractant was distributed in a pattern which resembled that seen with

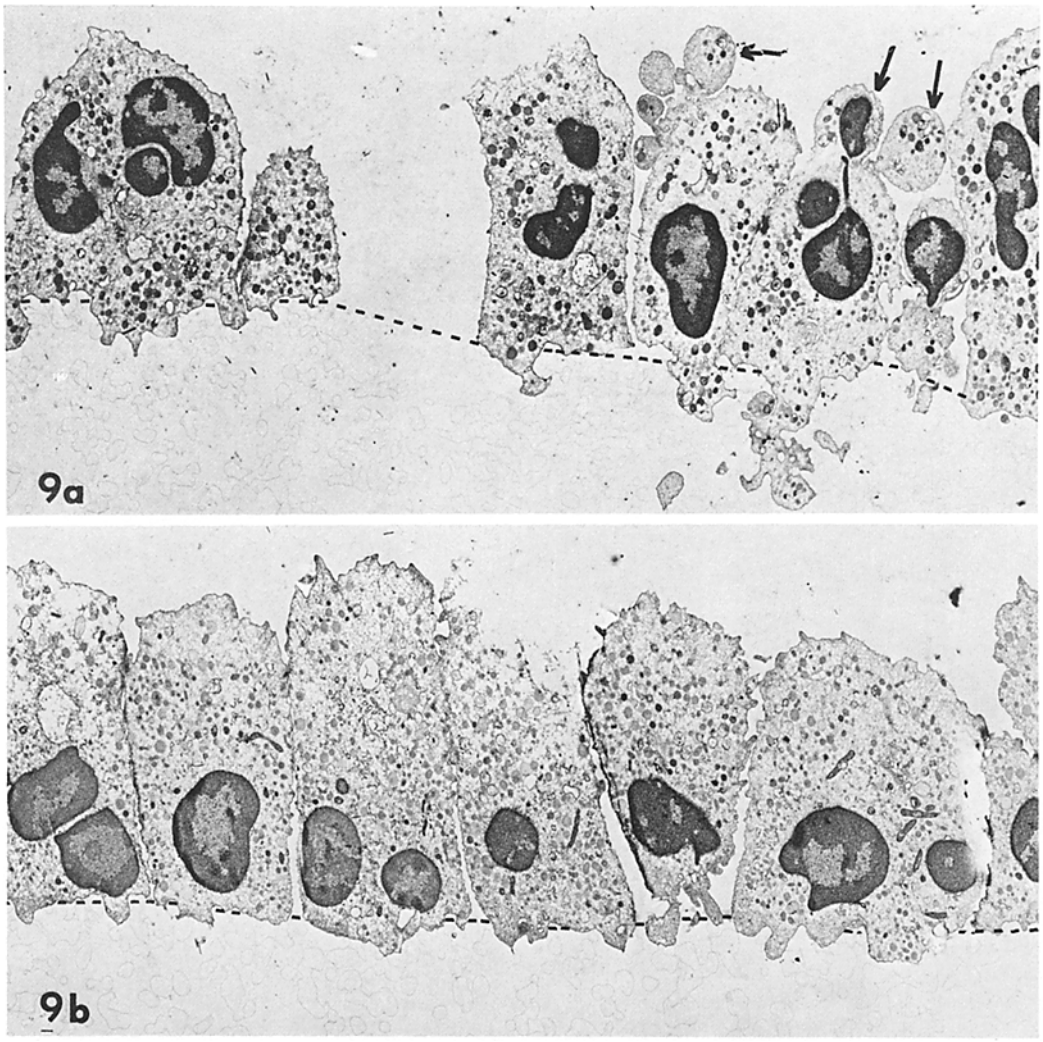

FIGURE 9 Composite electron micrograph of neutrophils oriented under conditions of directed migration on 0.45-μm pore filters and subsequently exposed to cytochalasin B, 3 μg/ml, either maintaining the 5% activated serum beneath the filter (*a*), or reversing the gradient by placing the 5% activated serum above the filter (*b*), as described in the text. Cytochalasin B also causes blebbing as seen in some cells in (*a*) (arrows). The filter surface is indicated by dashed lines. (*a*), (*b*) × 4,050.

activated random migration, in that a "peak" was no longer seen in the distribution curve (Fig. 14). However, the colchicine-treated cell population in a gradient of chemoattractant did migrate farther into the filter than was seen in activated random migration. This type of alteration is consistent with the hypothesis that colchicine primarily affects the cell's ability to maintain a fixed orientation during migration, and that microtubules are important, but not necessarily the sole means of maintaining the unidirectional migration.

Additional studies were done to further evaluate the significance of these observations. A lower concentration of colchicine was used (10^{-6} M), and another assay of cell locomotion was employed utilizing a double micropore filter system and ^{51}Cr-labeled leukocytes as described in Methods. As shown in Fig. 15, colchicine did not affect random migration with the radioassay.

However, as noted with the morphologic assay, both activated random and directed migration were significantly impaired.

Correlation of the Dose-Response Effects of Colchicine upon Neutrophil Structural Orientation, Microtubule Structure, and Directed Migration

Although colchicine has been shown to have effects upon the neutrophil other than antitubulin activity, many of these effects occur at concentrations greater than necessary to achieve antimitotic activity in other cell systems, and presumably antimicrotubule activity in the neutrophil (5). A close correlation of colchicine's dose-response effects upon neutrophil microtubule assembly, structural orientation, as well as directed migration in a chemoattractant gradient would constitute strong evidence for linking these effects with the antitubulin activity of colchicine. Cell viability was not affected by colchicine, except at 10^{-4} M where a 5% loss of viability was noted (trypan blue dye exclusion).

For morphological analyses, 0.45-μm pore filters were used. After a 45-min incubation, the filters with adherent cells were prepared for electron microscopy. Fig. 16*a*, *b*, and *c* shows cells in 10^{-8}, 10^{-7}, and 10^{-6} M colchicine, respectively. In 10^{-8} M colchicine, cells were indistinguishable from controls. The nuclei oriented to the rear of the cells, numerous pseudopods penetrated the filter pores, and high magnification analysis showed that microtubule, centriole, and microfilament orientations were normal. At 10^{-7} M colchicine, subtle differences from untreated cells were noted. Slight rounding of cells, loss of nuclear orientation, and decreased numbers of pseudopods in the filter pores were seen. At 10^{-6} M colchicine (Fig. 16*c*), slightly fewer cells adhered to the filter. The cells were rounder, and a dramatic loss of nuclear orientation and pseudopod penetration into the filter was seen. Some pseudopods entered the filter and then reemerged, suggesting an impairment of sustained orientation of the advancing pseudopod. High magnification description of these cells was previously noted (Fig. 12).

Nuclear orientation under conditions of directed migration was quantitated in the presence of increasing concentrations of colchicine and plotted in the upper part of Fig. 17. There was little difference in nuclear orientation at 10^{-8} M colchicine. However, from 5×10^{-8} thru 10^{-6} M colchicine, there was progressive loss of cell orientation until at 10^{-6} M the nuclear location was no different from midcell position. With increasing concentrations of colchicine, there was a significant trend toward increasing variability (SD) of nucleus location among cells (Fig. 17). Therefore, although the average nuclear position tended toward midcell location with high concentrations of colchicine, this actually reflected increased random distribution of nuclei within the cells.

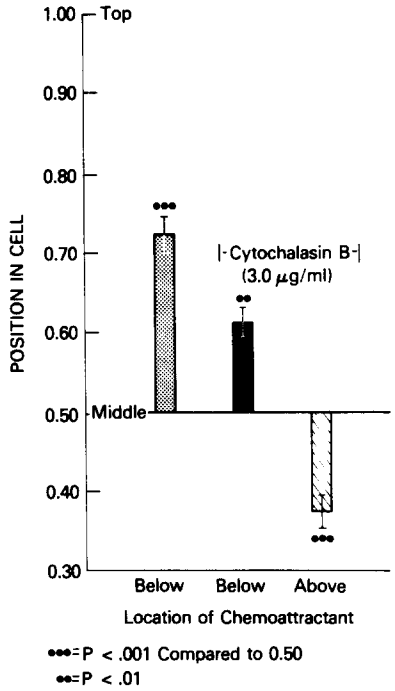

FIGURE 10 Average position of the nucleus in the vertical axis of neutrophils incubated under conditions of directed migration (left bar), or subsequently exposed to cytochalasin B (3 μg/ml) for 30 min with the gradient of chemoattractant maintained beneath the cells (middle bar) or reversed with gradient source above the cells (right bar). The graph is constructed as described for Fig. 2. Bars represent mean ± SEM, 30 determinations.

Cells were also examined by high magnification electron microscopy to determine the effect of increasing concentrations of colchicine on both the centriole position and the number of centriole-associated microtubules. As seen in Table III, significant orientation of centrioles was maintained at 10^{-7} M colchicine. However, a marked loss of centriole orientation was noted with 10^{-6} M colchicine.

The number of centriole-associated microtu-

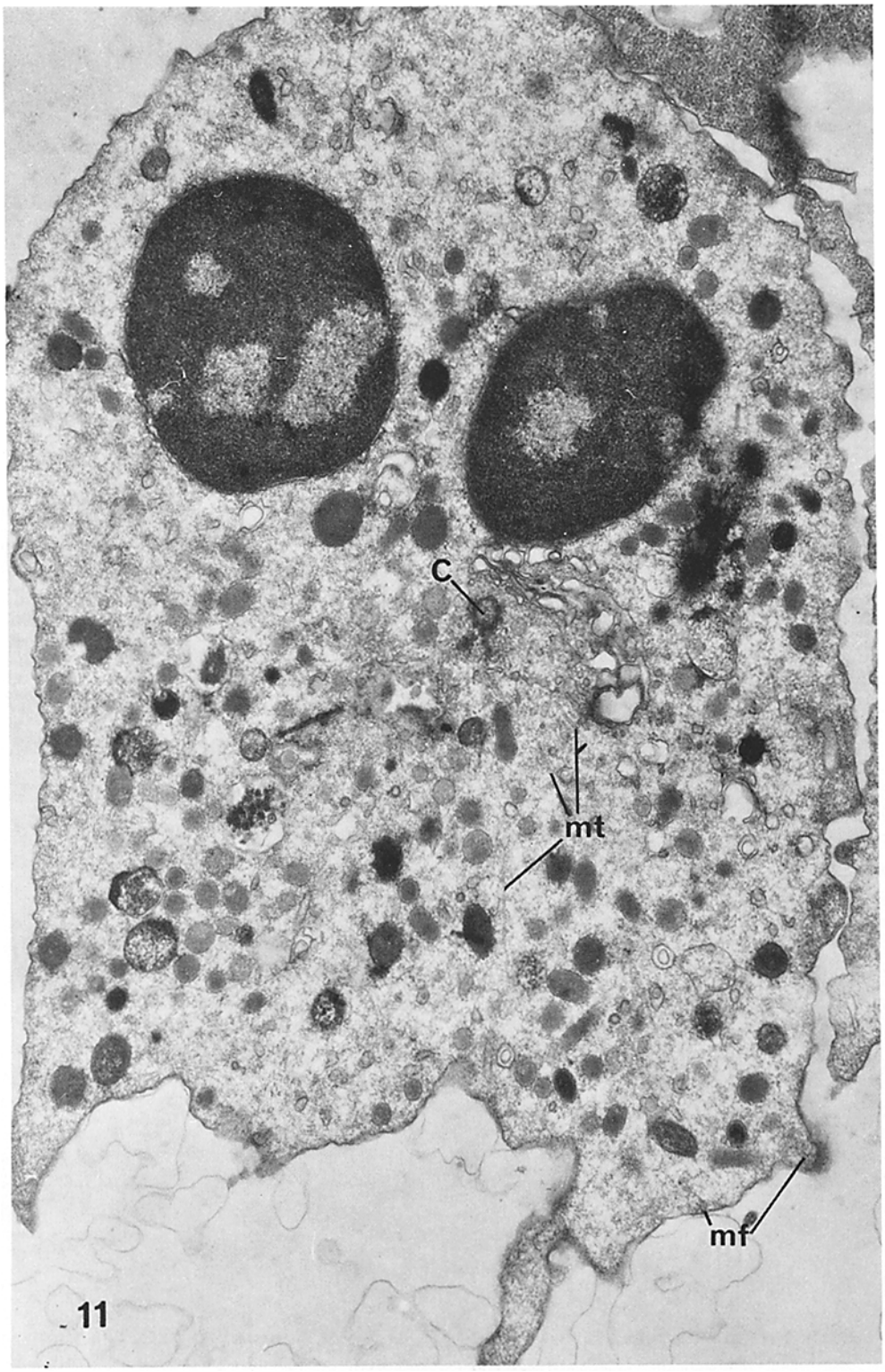

FIGURE 11 High magnification of a neutrophil oriented in a gradient of 5% activated serum and subsequently exposed to cytochalasin B (3.0 μg/ml) for 30 min, maintaining the chemoattractant beneath the cell. The centriole (*C*) can be seen just beneath the nucleus, segments of microtubules (*mt*) radiate out from the centriole, and a few collections of microfilaments (*mf*) are noted. × 19,500.

TABLE II

Effect of Cytochalasin B and Colchicine on Neutrophil Centriole Orientation During Directed Migration

Conditions of migration	No. of adjacent cells*	No. of cells in which the centriole was located	Position of centriole relative to nucleus (no. at each location)			Correlation of direction of gradient and mean score			
			above	middle	below	Mean score‡ ±SD‡	P§	χ^2	P, χ^2
Cytochalasin B (3 μg/ml) Chemoattractant below cells	15	10	0	0	10	−1.00 ± 0	<0.001		
								20.0	<0.001
Cytochalasin B (3 μg/ml) Chemoattractant above cells (30-min reverse)	16	10	10	0	0	+1.00 ± 0	<0.001		
Colchicine (10^{-6} M) Chemoattractant below cells	20	10	2	5	3	−0.10 ± 0.22	>0.6		
								1.85	>0.10
Colchicine (10^{-6} M) Chemoattractant above cells (5-min reverse)	18	8	4	3	1	+0.38 ± 0.25	>0.1		

* 15-20 serial sections extending through 1-1.5-μm depth were examined, using both photographs and freehand drawings to positively identify cells from one section to the next. Centriole position was determined in the vertical cell axis relative to nucleus position. See Methods.

‡ Mean Score = $\frac{\text{Number of centrioles above nucleus} - \text{number of centrioles below nucleus}}{\text{total number of centrioles located}}$

§ Significance level of difference between mean and 0, calculated from Z = mean score/SD, which was assumed to have a standard normal distribution.

bules was quantitated at the various concentrations of colchicine as outlined in Methods. The microtubules observed were in the same cell sections as those used to construct Table III for centriole orientation. Decreasing numbers of centriole-associated microtubules were noted at concentrations as low at 10^{-8} M colchicine, and these decreases were significant at 10^{-6} M (Fig. 18).

The dose-response of colchicine upon directed migration was determined by measuring the number of ^{51}Cr-labeled neutrophils traversing a top 3.0-μm filter and migrating into a lower 3.0-μm filter. Neutrophils were incubated with colchicine for 30 min at 37°C before the study. As shown in Fig. 19, 10^{-8} M colchicine had no effect on the ability of cells to migrate into the lower filter. However, at 10^{-7} M colchicine and higher concentrations, significant impairment of directed migration was noted.

Effects of Lumicolchicine

Lumicolchicine was used as a control for the colchicine studies at 10^{-6} M, well into the concentration range of colchicine effects. Under conditions of chemotaxis, lumicolchicine did not impair nuclear orientation (average nuclear position 0.75 ± 0.02, $P < 0.001$ as in Fig. 17) or microtubule counts (7.95 ± 1.11 as in Fig. 18). Lumicolchicine had no effect on leukocyte migration as assessed with both the morphological assay and the radioassay.

DISCUSSION

In the current study, we have shown that a gradient of chemoattractant induces a polarization of neutrophil structure and function that resembles that of an actively migrating cell even when migration is impeded by a physical barrier. This involves an increase of microfilaments at the leading end, with restriction of pseudopod formation to the leading end toward the chemoattractant. In addition, the centriole with associated microtubule array orients in the cell to the side of the nucleus toward the chemoattractant, and the nucleus is significantly shifted to the rear of the cell. A relatively rapid reorientation occurs when the direction of the chemoattractant stimulus is changed.

We have also defined some of the structural differences between the three types of neutrophil migration: random migration (cells in buffer), activated random migration (cells in uniform concentration of chemotactic factor), and directed migration or chemotaxis (cells in a gradient of chemotactic factor). The most striking morphological changes distinguishing the types of migration involve overall cell shape and orientation of specific

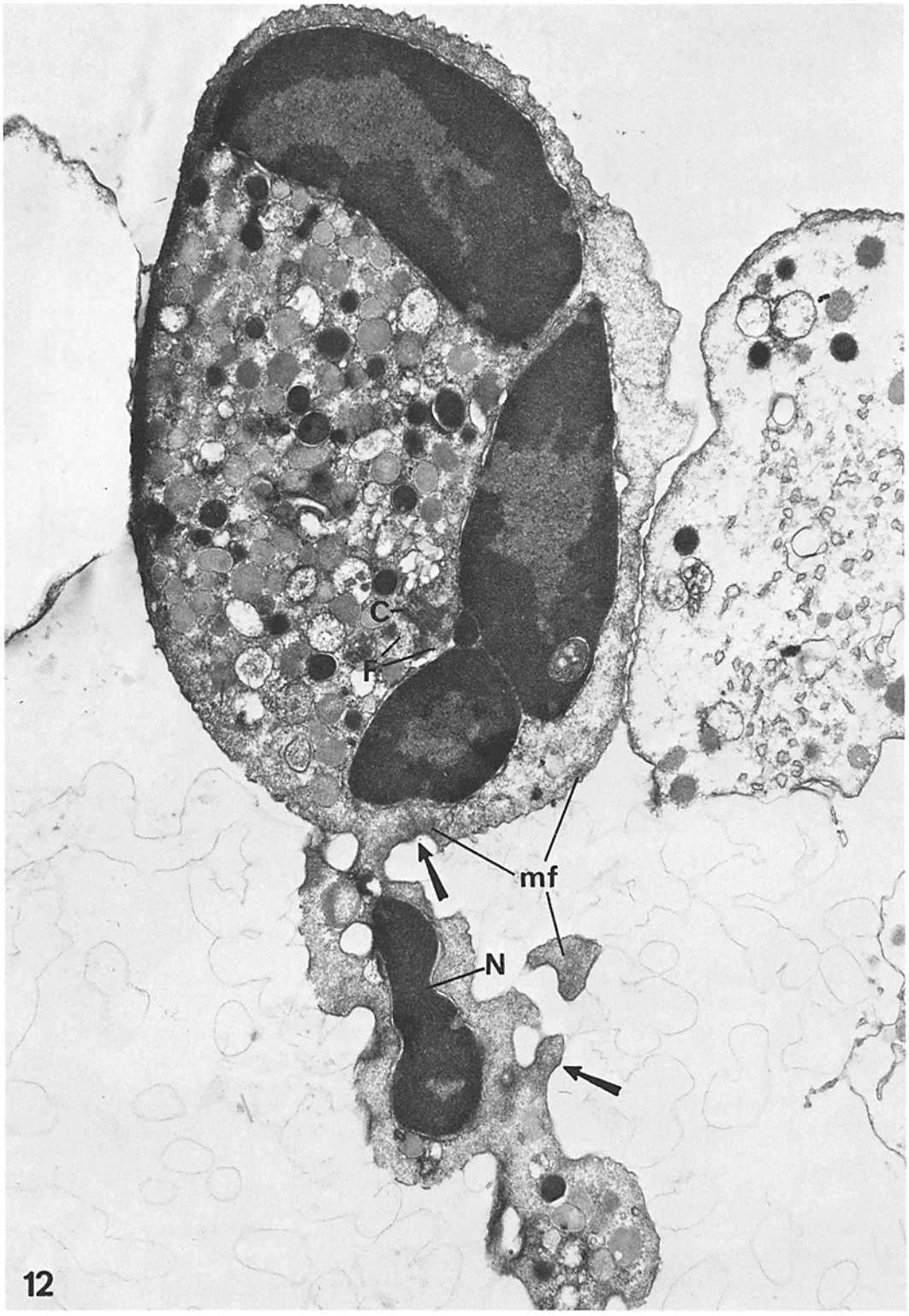

FIGURE 12 Colchicine-treated neutrophil under conditions of directed migration for 45 min. 5% activated serum is beneath the cells, and colchicine (10^{-5} M) is present on both sides of the filter. A portion of the nucleus (*N*) is in the pseudopod. The centriole (*C*) is located at the side of the nucleus. Microtubules are absent, but there is an increase of 100 Å diameter filaments (*F*) near the centriole. A thin layer of submembranous microfilaments (*mf*) is seen at the sides and top of the cell, and increased amounts of microfilaments including dense condensations (arrows) are seen in the pseudopod. × 15,900.

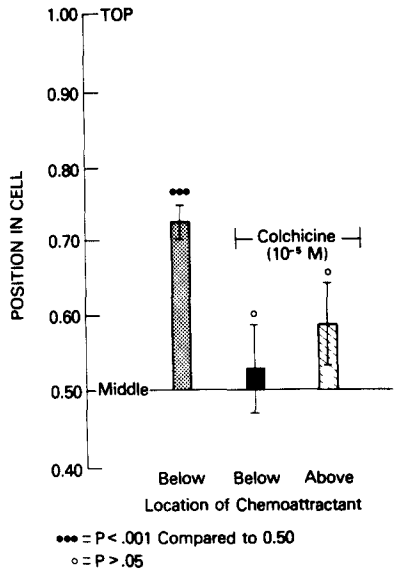

FIGURE 13 Average position of the nucleus in the vertical axis of colchicine-treated neutrophils incubated under conditions of directed migration in the presence of colchicine (10^{-5} M) with 5% activated serum beneath the cells (middle bar) or after 5-min reversal of the gradient (right bar). The average position of the nucleus of control cells under conditions of directed migration is included for comparison (left bar). The graph is constructed as described for Fig. 2. Bars represent mean ± SEM, 30 determinations.

intracellular structures, rather than the sudden appearance of any new structures.

When neutrophils were in buffer (random migration), many cells were rounded or moderately spread on the surface of the filter without evidence of pseudopod formation. In the presence of a uniform concentration of activated serum (activated random migration), an increased number of cells had a motile morphology (Fig. 1), and the population of cells migrated farther into a filter than randomly migrating cells (compare untreated cells in Fig. 14), thus confirming our earlier analysis of the effect of activated serum on the morphology of cells in suspension (12). Therefore, activated serum converted the cell population from a resting to migratory state. In addition, when applied as a gradient, activated serum oriented almost the entire population of cells. These observations do not necessarily imply that all chemotactic stimuli must increase the rate of locomotion; indeed, there is evidence that with some stimuli the individual cells orient but do not migrate faster when exposed to a gradient of chemotactic factor (18, 19). Instead, our morphologic observations indicate that the difference between random and directed migration is that in the latter orientation is uniform in almost the entire cell population.

We have previously proposed that the increased microtubule assembly we noted during neutrophil migration was critical for cell orientation (12). The demonstration of specific localization of the microtubule system in our current paper provides additional evidence in support of this hypothesis. Moreover, the sequence of events following reversal of the chemotactic stimulus gradient enables

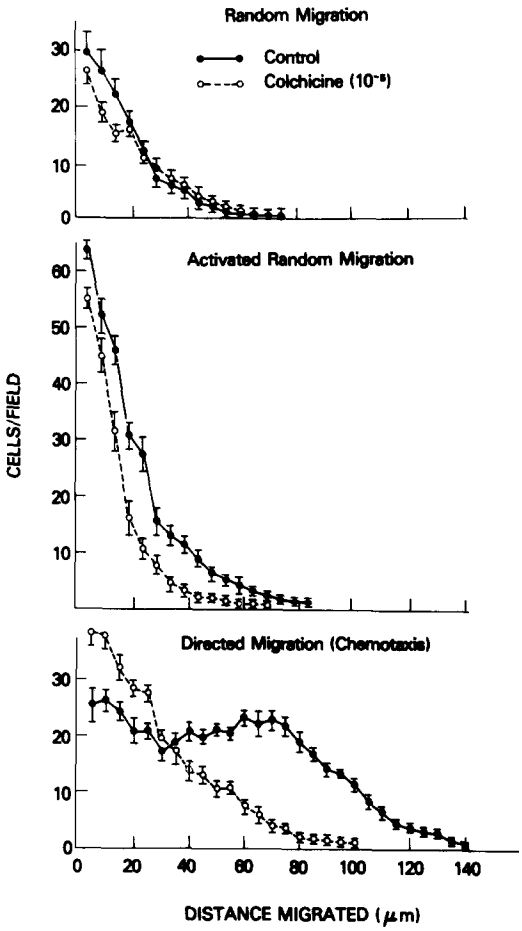

FIGURE 14 Effect of colchicine (10^{-5} M) upon the distribution of neutrophils within a 3.0 μm-pore size filter after 1 h of migration under conditions of random, activated random, or directed migration. The abscissa is the distance into the filter matrix from the top of the filter. The ordinate is the number of cells per high power field seen at each depth of focus into the filter. Each point represents the mean ± SEM of 10 determinations in duplicate filters (see Materials and Methods).

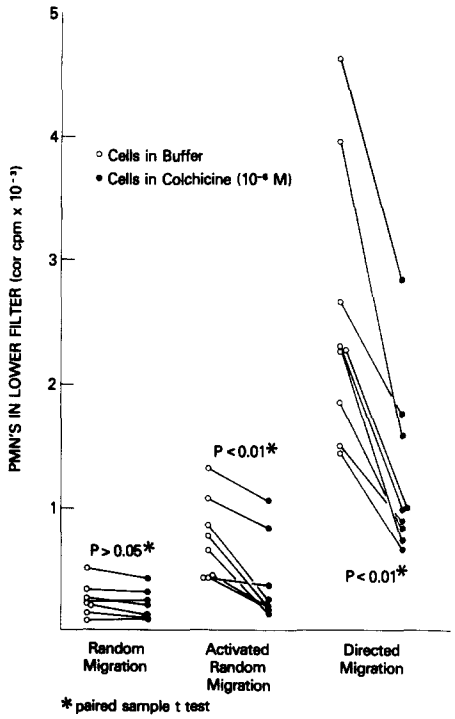

FIGURE 15 Effect of colchicine on the random, activated random, and directed migration of ^{51}Cr-labeled neutrophils (PMN, polymorphonuclear neutrophils) through a top and into a lower 3-μm filter. Each point is the mean of four replicate chambers, and lines connect individual experiments using cells from different donors.

additional speculation regarding the role of the various structural elements in stabilizing the direction of migration. It has been noted by several observers that most neutrophils migrating on a solid substrate respond to changes in the direction of a chemoattractant by describing a curve and maintaining the same leading end (1). Ramsey has noted that neutrophils subjected to a rapid change in the direction of chemoattractant to a position opposite the initial migration appear to resorb the leading pseudopod and produce a new one at what was previously the tail (20). Although the cell stops its forward motion immediately, Ramsey's report indicates that it takes 45 s of reestablishment of locomotory morphology in the opposite direction before migration is resumed in the new direction. The nature of the shift in the direction of pseudopod extension is not known. However, the initial paradoxical downward movement of the centriole away from the new source of the chemotactic gradient in our studies may reflect an inertial phenomenon with respect to the inside of the cell in its initial attempt to reorient toward the stimulus. In fact, this response was so strong that early after reversal, some cells spread out laterally just beneath the filter surface, with the entire cell, including the nucleus, enmeshed in the filter pores. In other cells lying on the filter, the centriole and nucleus were parallel to the filter surface as the nucleus relocated toward the bottom of the cell and the centriole relocated toward the top. The increase of microfilaments early after reversal in the side of the cell toward the chemoattractant was noted only when the centriole had completed relocation to a position above the nucleus. Pseudopod formation and upward migration of the cells into the fluid medium also was not seen before the time when relocation of centriole was evident in most cells. Thus, our observations not only support the concept that microtubules are required for maintaining directed migration but, in addition, that the position of the centriole and its associated array of microtubules are involved in establishing the direction of migration after a change in location of the stimulus.

Cytochalasin B and colchicine were used as chemical probes to further evaluate the relative roles of the cellular contactile apparatus and microtubules in neutrophil orientation and pseudopod formation during chemotaxis. Cytochalasin B at 3 μg/ml prevents neutrophil migration (3, 12, 14, 26), and in the current study it affected the amount of microfilaments seen, but did not prevent the maintenance of the internal orientation of the neutrophil in response to a gradient of chemoattractant. Thus, a normally functioning microfilament apparatus is not required for the orientation process. However, impairment of pseudopod penetration into the filter resulted from loss of microfilament function. Therefore, pseudopod formation appears to require normal microfilament function.

We have suggested that microtubules function to maintain orientation in migrating neutrophils. Our studies with colchicine and lumicolchicine were designed to further explore this possibility. Initial experiments showed that the internal orientation of the neutrophil, in response to a gradient of chemoattractant, was eliminated by colchicine treatment, and in the presence of colchicine, the nucleus and centriole occupied random positions within the cell. Our dose-response studies demonstrated that these effects of colchicine correlated with concentrations previously shown

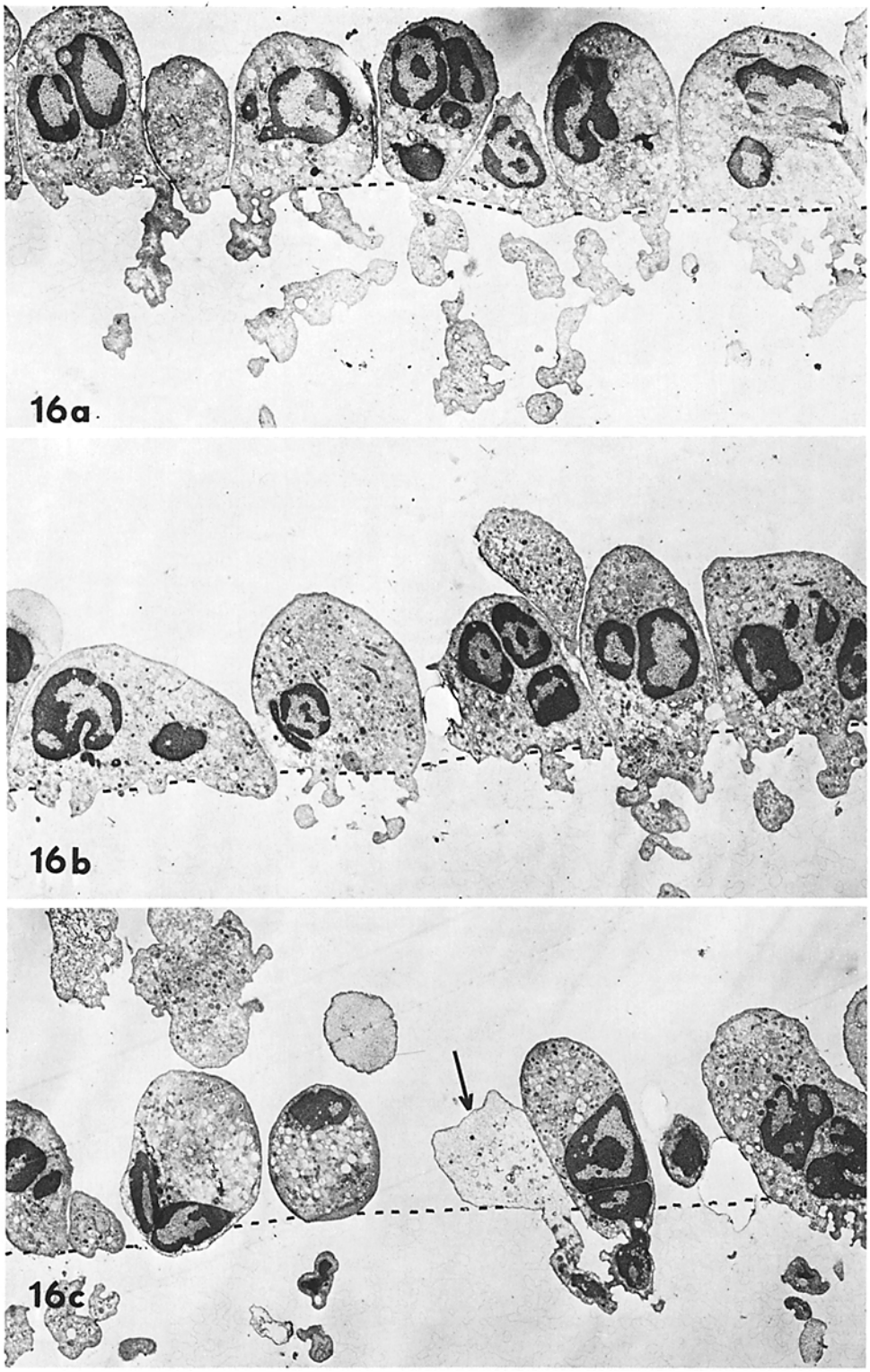

FIGURE 16 Composite electron micrograph of neutrophils oriented on a 0.45-μm pore filter under conditions of directed migration in the presence of colchicine 10^{-8} M (*a*), 10^{-7} M (*b*), and 10^{-6} M (*c*). The neutrophils were initially incubated for 30 min in suspension in the respective concentrations of colchicine, and then added to chemotactic chambers for 45 min. Note that at 10^{-6} M colchicine (*c*), the cells are rounded and the nuclei are randomly oriented within the cells. Pseudopods may enter the filter matrix, but occasionally reemerge (arrow). The dashed lines indicate the surface of the filter. (*a*) × 3,450, (*b*), (*c*) × 3,300.

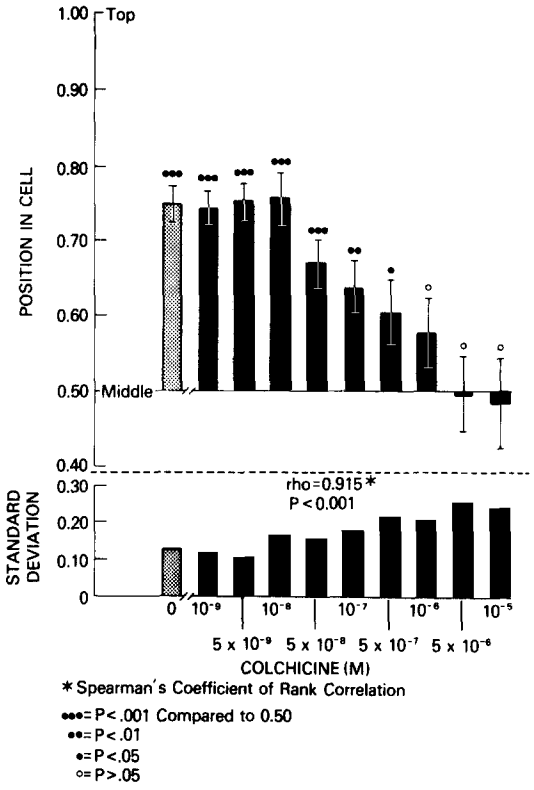

FIGURE 17 Dose-response effect of colchicine upon nuclear orientation under conditions of directed migration. The graph in the upper panel is constructed as described for Fig. 2, and represents the average position of the nucleus in the vertical axis of the neutrophils (mean ± SEM) of 30 determinations. The bars in the lower panel are the SD of the nuclear position at each colchicine concentration.

to inhibit microtubule assembly (15), and interestingly, in our study, they occurred at concentrations of colchicine that can be obtained in vivo (9). Lumicolchicine, an agent possessing many of the properties of colchicine, except tubuling binding activity (22), had no effect on these processes. Thus, microtubules are important in the initiation and maintenance of the internal structural orientation of the neutrophil. In untreated cells, the radial array of microtubules may prevent the random drift of the nuclear lobes by locking them into position on the side opposite the source of chemoattractant.

Colchicine also affected pseudopod formation, but in a somewhat complex manner. In the absence of colchicine, when the direction of the gradient of chemoattractant was reversed, neutrophils responded by extending pseudopods up into the fluid medium as they withdrew from the filter. In addition, when neutrophils were suspended in a chemotactic factor, without a substrate, they were also able to form pseudopods. Thus, in the absence of colchicine, pseudopod extension and orientation are not dependent on the location of a solid substrate. In the current study, colchicine, in concentrations that inhibited microtubule assembly, prevented the formation of pseudopods by cells in suspension, even in the presence of a strong stimulus such as activated serum. However, upon adherence to a substrate, colchicine-treated cells were capable of forming pseudopods, although they were narrow and elongated. However, unlike control cells, pseudopods did not develop on the nonsubstratum side of the cell when the stimulus source was reversed and placed above the cells. The formation of pseudopods and their

TABLE III

Dose-Response Effect of Colchicine on Neutrophil Centriole Orientation under Conditions of Directed Migration

Molar concentration of colchicine	No. of centrioles located by random survey	Position of centriole relative to nucleus (no. at each location)			Mean score*	P‡
		above	middle	below		
No colchicine	8	0	2	6	−0.75 ± 0.16	<0.01
10^{-9} M	11	0	2	9	−0.82 ± 0.12	<0.001
10^{-8} M	13	0	4	9	−0.69 ± 0.13	<0.001
10^{-7} M	14	1	3	10	−0.64 ± 0.17	<0.01
10^{-6} M	13	4	7	2	±0.15 ± 0.19	>0.4

* Mean score = $\frac{\text{Number of centrioles above nucleus} - \text{number of centrioles below nucleus}}{\text{total number of centrioles}}$

‡ Significance level of difference between mean and 0, calculated from Z = mean score/SD, which was assumed to have a standard normal distribution.

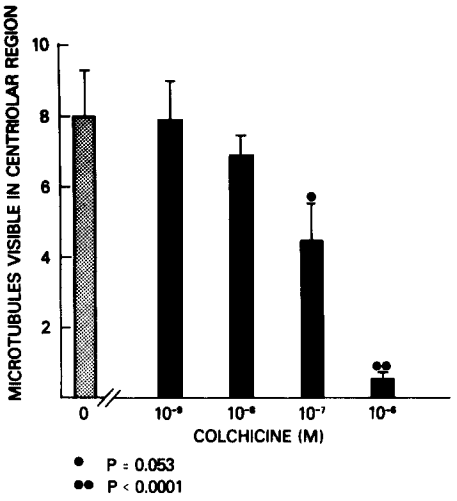

FIGURE 18 Dose-response effect of colchicine upon centriole-associated microtubules under conditions of directed migration. Bars represent mean ± SEM microtubule counts in 8–13 cells for each colchicine concentration.

orientation in the absence of microtubules appeared dependent upon a suitable surface for cells to adhere. The dense bundles of microfilaments noted in untreated cells at sites of attachment to the filter surface were unaffected by colchicine treatment. This microfilaments system probably contributes to the formation and orientation of pseudopods when a filter substratum is provided as an exoskeleton to guide cell movement. However, the microfilament-filter substrate combination is an imperfect substitute for the microtubule skeleton and, in the absence of microtubules, pseudopod formation and activated random migration, as well as directed migration, are significantly impaired.

It is possible to formulate a tentative structural model of neutrophil migration and chemotaxis based upon the findings of our studies. Microtubules appear to be the primary organizers of the internal contents of the neutrophil. They modulate pseudopod formation by providing a cytoskeleton within the core of the pseudopod that is linked to the internal portions of the cell body by the nature of the radial structure of the microtubule-centriole apparatus. In this way, pseudopod extension is possible in the absence of adhesion to a substrate, and its location on the cell body is stabilized.

During chemotaxis, the maintenance of pseudopod orientation is probably critical for efficient migration toward the stimulus. However, our data do not suggest that enhanced forward migration under conditions of chemotaxis is entirely eliminated by colchicine treatment. As was noted in Results, there is also a small impairment of activated random migration by colchicine, the explanation for which may be found in previous observations of various cell types treated with colchicine. Randomly migrating cells treated with colchicine show an increased tendency for eccentric and more frequent direction changes, and also a decreased rate of peripheral spread of a cell population (1). Thus, stabilization of the internal structure and consistent organization of the leading pseudopod may also be necessary for efficient random migration.

Based upon the findings in this study, we propose a model in which microtubules modulate pseudopod formation and orientation, and enhance migration in a single direction. However, these events are probably also under the influence of other systems, including the microfilament network. The biochemical basis for controlling these processes is not known, although a role for calcium has been speculated (12, 16). Future experiments are planned to explore the molecular basis for the orientation of microtubules and microfilaments during neutrophil chemotaxis.

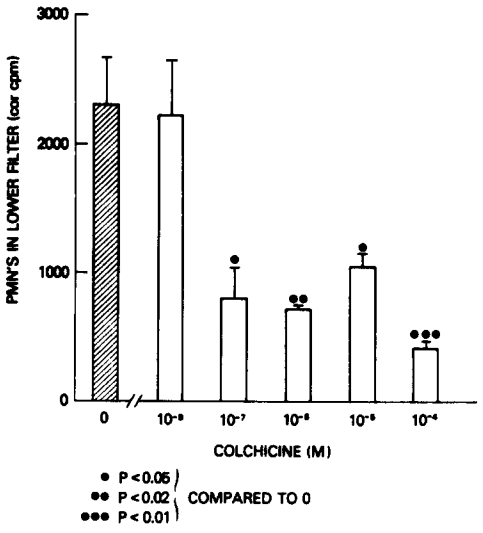

FIGURE 19 Dose-response effect of colchicine upon directed migration as determined by the number of ^{51}Cr-labeled neutrophils traversing a top and penetrating a lower 3.0-μm filter. Each bar represents the mean ± SEM of four determinations.

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