LOCALIZATION OF SUBMEMBRANOUS CATIONS
TO THE LEADING END OF HUMAN NEUTROPHILS
DURING CHEMOTAXIS

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ABSTRACT

Potassium pyroantimonate was used to localize sites of bound cations in human
neutrophils under conditions of random migration, stimulated random migration
(chemokinesis), and directed migration (chemotaxis). The cells were placed in a
standard chamber in which 0.45-μm micropore filters separated the cells from the
stimulus (buffer, Escherichia coli endotoxin-activated serum or the synthetic
chemotactic peptide N-formyl-Met-Leu-Phe). The small pore filters permitted
pseudopod formation but impeded cell migration through the filter. Cells exam-
ined under all conditions had electron-dense precipitates of antimonate salts in
some granules. However, antimonate deposits were localized in the condensed
chromatin of the nucleus during random migration and associated to a large
extent with the uncondensed nuclear chromatin during chemokinesis and chem-
otaxis. Under conditions of chemokinesis deposition of antimonate precipitates
appeared on the cytoplasmic side of the plasma membrane of neutrophils whereas
under conditions of chemotaxis cation deposits beneath the cell membrane were
localized to the pseudopods which were directed toward the chemoattractant. In
addition to endotoxin-activated serum, concentrations of N-formyl-Met-Leu-Phe
which caused neutrophil chemotaxis (10^{-8} M) also caused cation deposition
beneath the cell membrane at the leading end of the cell regardless of whether
albumin was present in the incubation media. However, with higher concentrations
of the synthetic peptide (10^{-5} M) which caused granule release and were not
chemotactic, submembranous cation deposition was not seen. EDTA (10 mM)
and EGTA (10 mM) removed nuclear, granular, and submembranous cation
deposits from neutrophils examined under conditions of chemotaxis. X-ray micro-
probe analysis of antimonate deposits revealed the possible presence of calcium
but did not detect sodium or magnesium. The data indicate that chemotactic
factors induce submembranous deposition of cations, most likely Ca^{++}, which
localize to the leading edge of cells exposed to a gradient of chemoattractant.

It is well known that cations are required for granulocyte motility (3, 14), and studies with ^{45}Ca have demonstrated a rapid calcium uptake (5, 20) and release (14, 20) when chemotactic factors interact with human neutrophils in suspension. In addition, we have shown previously that interaction of neutrophils with chemotactic factors significantly decreases the cell negative surface charge (12). As part of our studies to relate cations to cell locomotion, we recently presented preliminary findings indicating that cations localize beneath the plasma membrane at the leading end of neutrophils during chemotaxis but not during random migration or activated random migration (chemokinesis) (7, 8, 13). In this communication, we describe these findings in detail, provide data implicating the presence of calcium in the precipitates, and demonstrate that cells undergoing chemokinesis also accumulate cations beneath their cell membrane although not localized to one region of cells as in chemotaxis. This phenomenon, which is independent of the presence of albumin in the incubation media, can be caused by more than one chemotactic factor and is dependent on the concentration of the chemoattractant.

MATERIALS AND METHODS

Preparation of Neutrophils

Heparinized whole venous blood was obtained from healthy, adult volunteers and separated into a granulocyte-rich fraction by Hypaque (Winthrop Laboratories, New York)-Ficoll (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) and Dextran sedimentation (Pharmacia) techniques (6). Residual erythrocytes were eliminated by hypotonic lysis. This procedure routinely resulted in a cell fraction containing >95% neutrophils with 95% viability as determined by exclusion of trypan blue dye (Grand Island Biological Co., Grand Island, N. Y.) (21).

Neutrophils were suspended in Gey's balanced salt solution containing 2% bovine serum albumin, penicillin, and streptomycin (Gey's Medium, Microbiological Associates, Walkersville, Md.) at 2.5 × 10^6 cells/ml before use. In some experiments, cells were suspended in the same media without albumin.

Chemotactic Factors

Endotoxin-activated serum was prepared by incubating 30 μg of Escherichia coli endotoxin 0127:B8 lipopolysaccharide B (Difco Laboratories, Detroit, Mich.) with 0.1 ml of fresh serum in 0.9 ml veronal buffer (0.07 mM Ca^{++}, 0.5 mM Mg^{++}) for 1 h at 37°C. The solution was then heated for 30 min at 56°C to terminate the reaction and diluted 1:1 with Gey's medium before use (10).

Synthetic chemotactic peptide N-formyl (f)-Met-Leu-Phe (courtesy of Elliot Schiffmann, National Institute of Dental Research, National Institutes of Health, Bethesda, Md.) was used in concentrations varying from 10^{-5} to 10^{-10} M in Gey's medium with and without albumin.

Neutrophil function

Chemotaxis was evaluated using a ^{51}Cr (Amersham/Searle Corp., Arlington Heights, Ill.) radioassay (11) or a morphologic assay (33) which measures the number of cells migrating varying depths into a 3-μm cellulose nitrate filter. Lysozyme activity, β-glucuronidase, and lactic dehydrogenase were all assayed as described previously (4, 27, 29, 31) in incubation media or in whole-cell lysates prepared by cell disruption with 0.2% Triton X-100 (Rohm and Hass Co., Philadelphia, Pa.).

Preparation of Cells for Cation Localization by Electron Microscopy

The chemotactic chamber was divided into an upper and lower compartment by a 13-mm 0.45-μm micropore filter (Millipore Corp., Bedford, Mass.). This filter allows diffusion of soluble molecules and permits pseudopod penetration and cell orientation under conditions of chemotaxis but prevents cell migration through the filter (17). Neutrophils (0.8 ml containing 2.3 × 10^6 neutrophils/ml) were added to the upper compartment and incubated for 45 min at 37°C under conditions of random migration (buffer in upper and lower compartment), chemokinesis (chemoattractant in upper and lower compartments), or directed migration (buffer in upper compartment, chemoattractant in lower compartment). In other experiments, the effect of length of incubation (5, 15, 30, 45, and 90 min) on chemotaxis, cell orientation, and cation localization was investigated. In all cases, experiments were repeated at least three times and, in some cases (conditions of chemotaxis, incubation time 45 min), as many as 20 times. Within each experiment, ~100-200 cells were examined.

Preliminary studies were performed to determine the best fixation procedure for cation localization. At the end of the incubation, the fluid was aspirated from both compartments of the chemotactic chambers and immediately replaced with fixative. The filters were then removed and fixed for 1 h at room temperature or in the cold (ice bath). The neutrophils were either fixed in 2% osmium tetroxide (Electron Microscopy Sciences, Fort Washington, Pa.) with 2% potassium pyroantimonate (Lot No. 730312, Fisher Scientific Co., Pittsburgh, Pa. (pH 7.2-7.8) (15, 16, 26) or prefixed, either in 2% glutaraldehyde (Electron Microscopy Sciences) in 0.1 M phos-

phate buffer (pH 7.3–7.8) followed by a 0.1 M phosphate buffer wash (pH 7.3–7.8) or in 2% glutaraldehyde in 0.1 M collidine buffer with 10 mM calcium (pH 7.3) and then washed with 0.1 M collidine buffer containing 10 mM calcium (pH 7.3). Neutrophils prefixed in glutaraldehyde, regardless of the buffer, its pH, or temperature, contained fewer antimonate deposits than those fixed directly in osmium-pyroantimonate. In addition, when calcium was added to the collidine buffer, precipitate did, not occur within secretory granules but did increase nonspecifically on the cell membrane. Maximum precipitation of antimonate was obtained when cells were fixed directly in alkaline (pH 7.8) osmium-pyroantimonate in the cold (ice bath). After fixation, cells were dehydrated with ethanol, and propylene oxide was added slowly to the ethanol (17). This solution was replaced with propylene oxide, and the filters were transferred to embedding molds and embedded in Epon 812. Thin sections were cut on an LKB ultramicrotome III (LKB Instruments, Inc., Rockville, Md.), and unstained sections or those sections stained with uranyl acetate and lead citrate were examined with a Philips 300 or a JEM 100C electron microscope. In some experiments, the pyroantimonate technique did not form electron-dense deposits; however, this was only seen when the shelf life of the pyroantimonate exceeded 2 yr. Deposition of precipitates in granules was used as a positive indicator that the histochemical procedure worked. When this criterion was met, the localization of cation deposits associated with the cell membrane and nucleus within experimental groups was always consistent and the overwhelming majority of cells reacted identically.

Analysis of Cation Deposits

Grids containing unstained thin sections of neutrophils which were incubated under conditions of chemotaxis and then fixed with 2% osmium-2% pyroantimonate (pH 7.8) were placed in a solution of 10 mM EDTA (Fisher Scientific Co.) or 10 mM EGTA (Fisher Scientific Co.) in 20 mM acetate buffer (pH 7.5) to determine whether the electron-dense precipitates contained calcium, magnesium, or sodium (26). Control experiments with 20 mM acetate buffer alone, pH 7.5, were also undertaken to insure that removal of the precipitate was a result of the chelating properties of EDTA and EGTA. After 3 h of agitating the solutions, the grids were removed, dried, and examined with the electron microscope.

In an effort to further characterize the cations in the electron-dense precipitates which occur along the inside of the pseudopod plasma membrane, x-ray microprobe analysis was performed on these deposits. Unstained 100- to 200-nm thick sections mounted on Formvar carbon-coated copper and nickel grids were probed at JEOL Laboratories (Medford, Mass.) by Mr. David Harling and at New York Medical College (Valhalla, N. Y.) by Dr. Tamido Sato with a JEM 100C in the stem mode, in conjunction with a Kevex x-ray energy dispersive

spectrometer model 5100 (Kevex Corp., Foster City, Calif.). An accelerating voltage of 80 kV, a filament current of 18 μA, and specimen tilt of 25° were used. In both cases, the x-ray spectra were collected using the 0–8 cV range for 100–300 s, using a spot (20 Å) or square probe (4,000 Å). The raw data were analyzed statistically by background subtraction. Attempts were made to separate interfering peaks, such as antimony, at Dr. Sato's laboratory.

RESULTS

Neutrophil Locomotion

Neutrophils in buffer (conditions of random migration) appeared primarily round (Fig. 1), although in some cells pseudopod formation was observed. When these cells were processed for cation localization, precipitates were found in the condensed chromatin of the nucleus and associated with some of the lysosomal granules (Fig. 2). The granules containing antimonate deposits were of varying sizes and shapes. Occasionally, deposits were observed on the outside surface of the plasma membrane.

Under conditions of chemokinesis, with equal concentrations of the synthetic peptide f-Met-Leu-Phe in the upper and lower compartments of the chemotactic chambers, cells showed increased asymmetry (Fig. 3). Precipitates of antimonate salts were observed in some granules and associated with some of the granule membranes. However, deposits were seen primarily in the uncondensed chromatin (Fig. 4, inset) and along the inside surface of the cell membrane when cells were incubated with 10^{-9} or 10^{-10} M concentrations of synthetic peptide in both chambers (Fig. 4). At higher concentrations of f-Met-Leu-Phe (10^{-8}–10^{-5} M), nuclear and cell membrane precipitate did not occur.

When cells were stimulated by a gradient of chemotactic factor (chemotaxis), there was a marked polarization of neutrophil morphology. The neutrophils were oriented with their pseudopods extending into the filter toward the chemoattractant and with the nucleus toward the rear of the cell (Fig. 5). Beneath the cell membrane of the pseudopods at the leading end of the cell, small electron-dense deposits were observed (Fig. 6). These precipitates followed the contour of the pseudopods and were associated with the cytoplasmic side of the plasmalemma (Fig. 6, inset). Precipitates of antimonate salts in the nuclei were no longer associated primarily with condensed chromatin as in random migration but were more
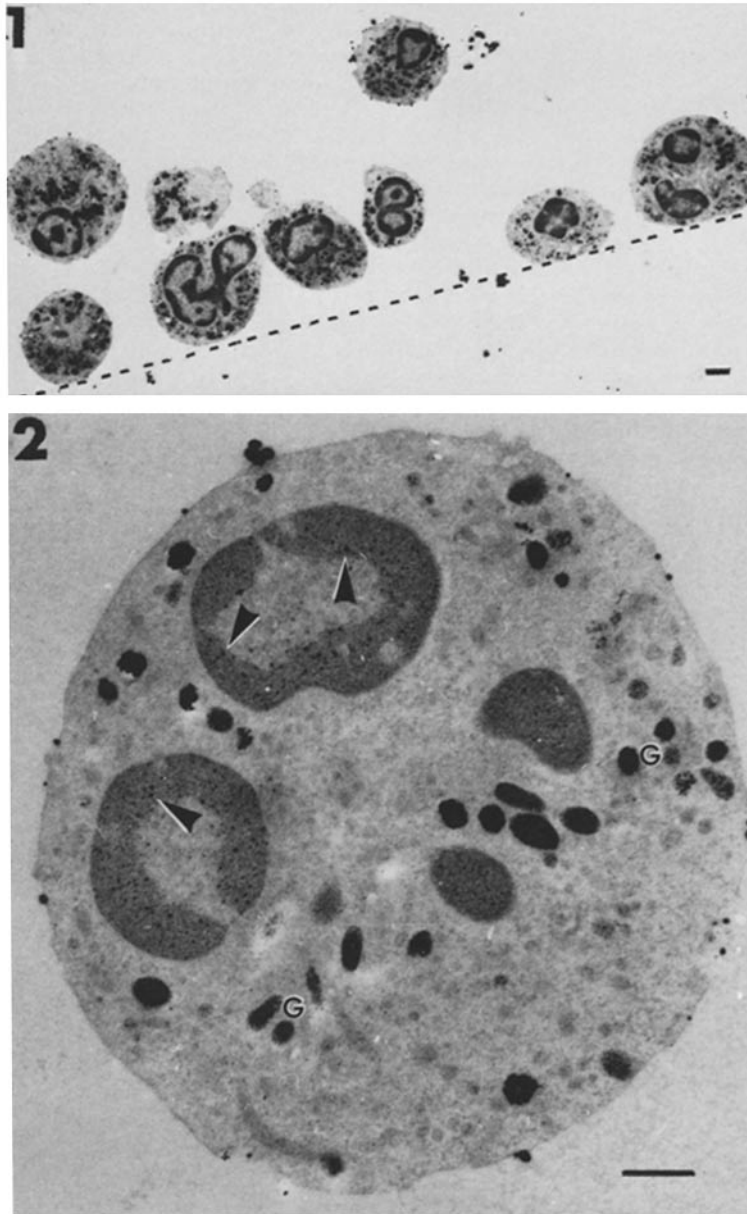
FIGURES 1 and 2 Human neutrophils under in vitro conditions of random migration (Gey's balanced salt solution in upper and lower compartments) fixed in osmium-antimonate. Under these conditions (Fig. 1), the cells appear rounded and either lie above or adhere to the surface of a 0.45-μm micropore filter. In an unstained section (Fig. 2), at higher magnification, electron-dense deposits of antimonate-cation complexes are seen in the condensed chromatin (arrowheads), in some granules (G), and occasionally on the outside of the plasma membrane. Fig. 1, uranyl acetate and lead citrate, × 1,800. Fig. 2, unstained, × 10,300. Bar, 1.0 μm.

commonly observed associated with the uncondensed chromatin as in chemokinesis. As noted with random migration and chemokinesis, electron-dense precipitates were also associated with some cytoplasmic granules (Fig. 6). Neutrophils which did not adhere to the filter remained round
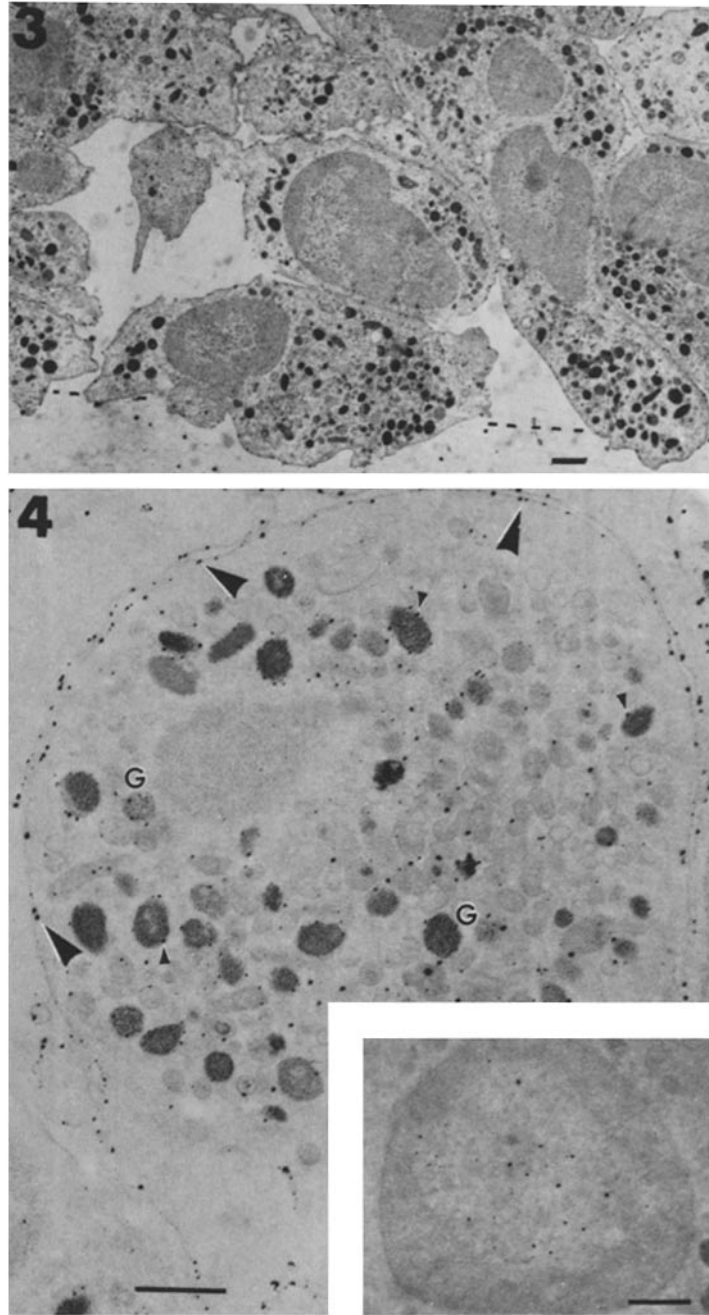
FIGURES 3 and 4   Human neutrophils under in vitro conditions of chemokinesis 10^{-9} M synthetic peptide, f-Met-Leu-Phe, in upper and lower compartments) fixed in osmium-antimonate. Many cells appear irregular with random pseudopod formation (Fig. 3). In an unstained section at higher magnification (Fig. 4), electron-dense deposits of antimonate-cation complexes are seen in some granules (G), associated with some granule membranes (small arrowheads) in the uncondensed chromatin (*inset*), and on the cytoplasmic side of the plasma membrane of the neutrophil (large arrowheads). Fig. 3, uranyl acetate and lead citrate, × 4,300. Fig. 4, unstained, × 13,200; *inset* unstained, × 9,300. Bar, 1.0 μm.

0.5μm

0.5μm

0.1μm
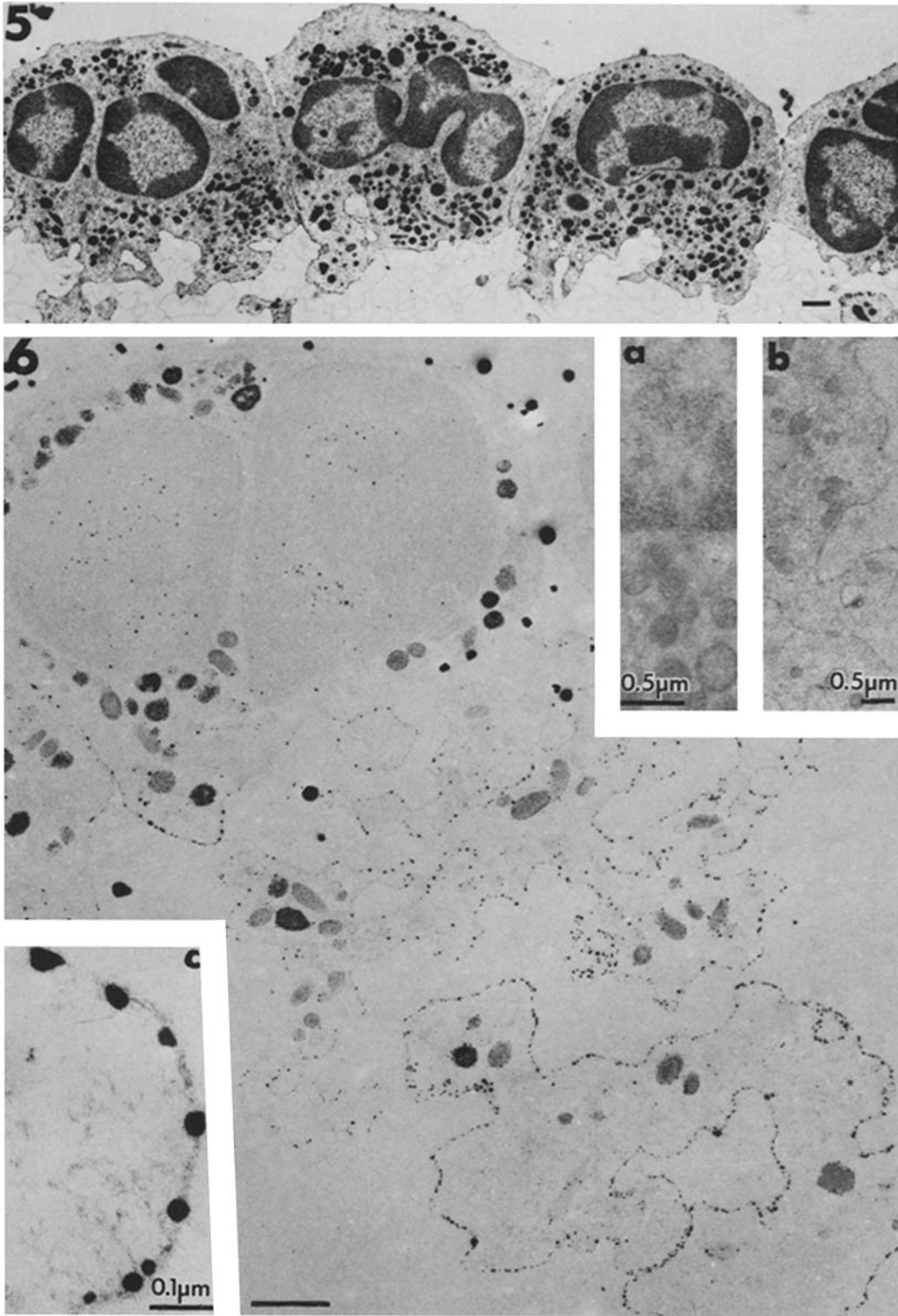
and did not have cation deposits beneath the cell membrane.

The ability of the neutrophils to orient in a chemical gradient was time dependent. When the neutrophils were incubated for 15–30 min, few settled out of the medium and oriented on the filter surface. Cells studied during these early time periods showed only slight pseudopod formation and no antimonate deposits beneath the plasma membrane closest to the chemoattractant. However, cells incubated for 45 or 90 min were oriented with pseudopods extending toward the chemoattractant, and cation deposits were observed beneath the cell membrane of the pseudopods.

N-f-Met-Leu-Phe Stimulation of Chemotaxis and Lysozyme Release

Various concentrations of the synthetic peptide, N-f-Met-Leu-Phe, have been reported previously to induce neutrophil chemotaxis and/or lysozyme release from cells migrating into cellulose nitrate filters (1, 2, 25). In our experiments, maximum chemotaxis occurred with 10^{-8} M N-f-Met-Leu-Phe; no chemotaxis was detected with concentrations below 10^{-10} M or above 10^{-6} M. Lysozyme release occurred with higher concentrations of N-f-Met-Leu-Phe with maximum release at 10^{-5} M, (Fig. 7). These findings are similar to earlier studies in rabbit peritoneal exudate neutrophils (25). No lactate dehydrogenase and <10% total cellular β-glucuronidase were released under these conditions.

Morphologic studies were performed to assess antimonate deposition in neutrophils using concentrations of N-f-Met-Leu-Phe which were optimal for chemotaxis (10^{-8} M) or lysozyme release (10^{-5} M). Neutrophils stimulated with synthetic peptide at concentrations of 10^{-8} M had electron-dense precipitates at the leading end of the cell on the cytoplasmic side of the cell membrane of the

pseudopods (Fig. 8). In addition, antimonate deposits were again noted in the uncondensed chromatin of the nucleus and associated with some of the granules. Both morphological orientation and cation deposits occurred in the presence or absence of bovine serum albumin in the incubation media. When a higher concentration of synthetic peptide (10^{-5} M) was used as the chemoattractant, granule exocytosis was observed at the leading end of the cell but precipitates of antimonate salts were no longer seen beneath the pseudopod cell membrane (Fig. 9). Granule exocytosis was observed only in the region of the cell in contact with the micropore filter.

The electron-dense deposits were removed from the nucleus, granules, and pseudopod cell membrane of neutrophils incubated under conditions of chemotaxis by 10 mM EDTA and 10 mM EGTA in acetate buffer at pH 7.5 (Fig. 6, inset). If the sections were washed in acetate buffer (pH 7.5) alone, the precipitate was not removed.
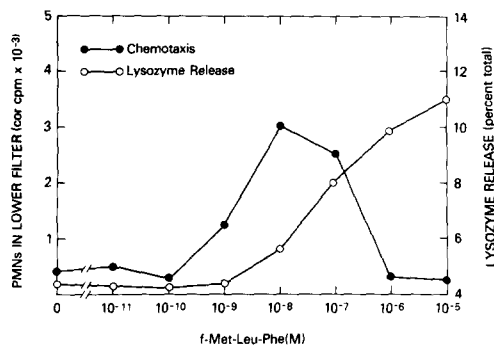
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FIGURE 7 Effect of various concentrations of the synthetic peptide, N-f-Met-Leu-Phe, on chemotaxis and secretion of lysozyme from ^{51}Cr-labeled human neutrophils migrating through two 3.0-μm micropore cellulose nitrate filters. Each point is the mean of four determinations.

FIGURES 5 and 6 Human neutrophils under in vitro conditions of chemotaxis (Gey's balanced salt solution in upper compartment, E. coli endotoxin-activated serum in lower compartment) fixed in osmium-antimonate. Under these conditions (Fig. 5), the neutrophils line up adjacent to each other with the nucleus at the rear of the cell and pseudopods extending into the filter toward the chemoattractant. At higher magnification (Fig. 6), electron-dense precipitates of antimonate salts are seen associated with the cytoplasmic side of the pseudopod membrane at the leading end of the cell (arrows), and at higher magnification, in the inset c. Deposits are also present in granules, on some granules membranes, and associated with the uncondensed chromatin. Antimonate salt precipitates were removed from the pseudopod cell membranes, granules, and nucleus of cells washed in EDTA (inset a) or EGTA (inset b). Fig. 5, uranyl acetate and lead citrate, × 3,800. Fig. 6, was unstained, × 11,500; inset a, × 20,000; inset b, × 12,500; inset c, × 100,000. Bar, 1.0 μm; bar, insets a and b, 0.5 μm; bar inset c, 0.1 μm.
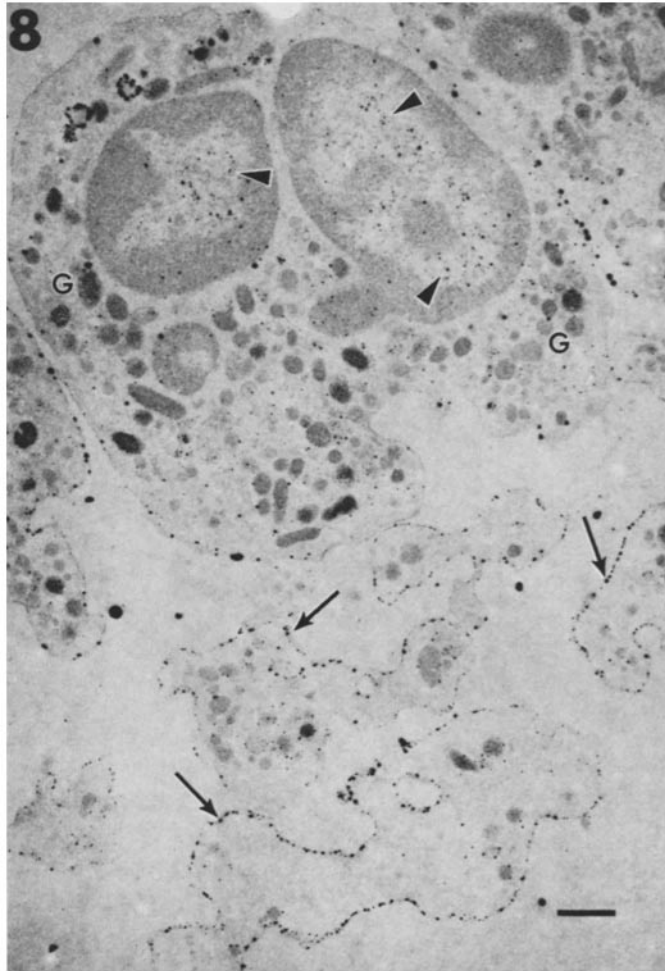
FIGURE 8 Unstained section of a human neutrophil under conditions of chemotaxis (Gey's balanced salt solution upper compartment, N-f-Met-Leu-Phe 10^{-8} M in lower compartment) fixed with osmium-antimonate. At this concentration of synthetic peptide, the cells are actively undergoing chemotaxis and antimonate-cation precipitates line the cytoplasmic side of the pseudopod cell membrane (arrows). Deposits are also seen in granules (G) and associated with the uncondensed chromatin (arrowheads), although some deposit is still seen associated with the condensed chromatin. Unstained, × 7,600. Bar, 1.0 μm.

X-ray microprobe analysis, by both Mr. David Harling and Dr. Tamiko Sato, of cation antimonate deposits at the leading end of neutrophils stimulated to undergo chemotaxis with activated serum showed spectra for osmium, chloride, antimony, and either nickel or copper, depending on the type of grid used to supply the sections. Sodium, potassium, magnesium, and manganese were not present in detectable amounts. The antimony peaks surrounded the calcium peaks and so it was not possible to verify the presence of calcium. Attempts by Dr. Sato to detect calcium by antimony subtraction were inconsistent and as a result were inconclusive.

DISCUSSION

The pyroantimonate technique is a specialized cytochemical procedure which can demonstrate the sites of critical concentrations of bound organic or inorganic cations as insoluble electron-dense pyroantimonate precipitates. Using this technique, we have demonstrated that the primary sites of
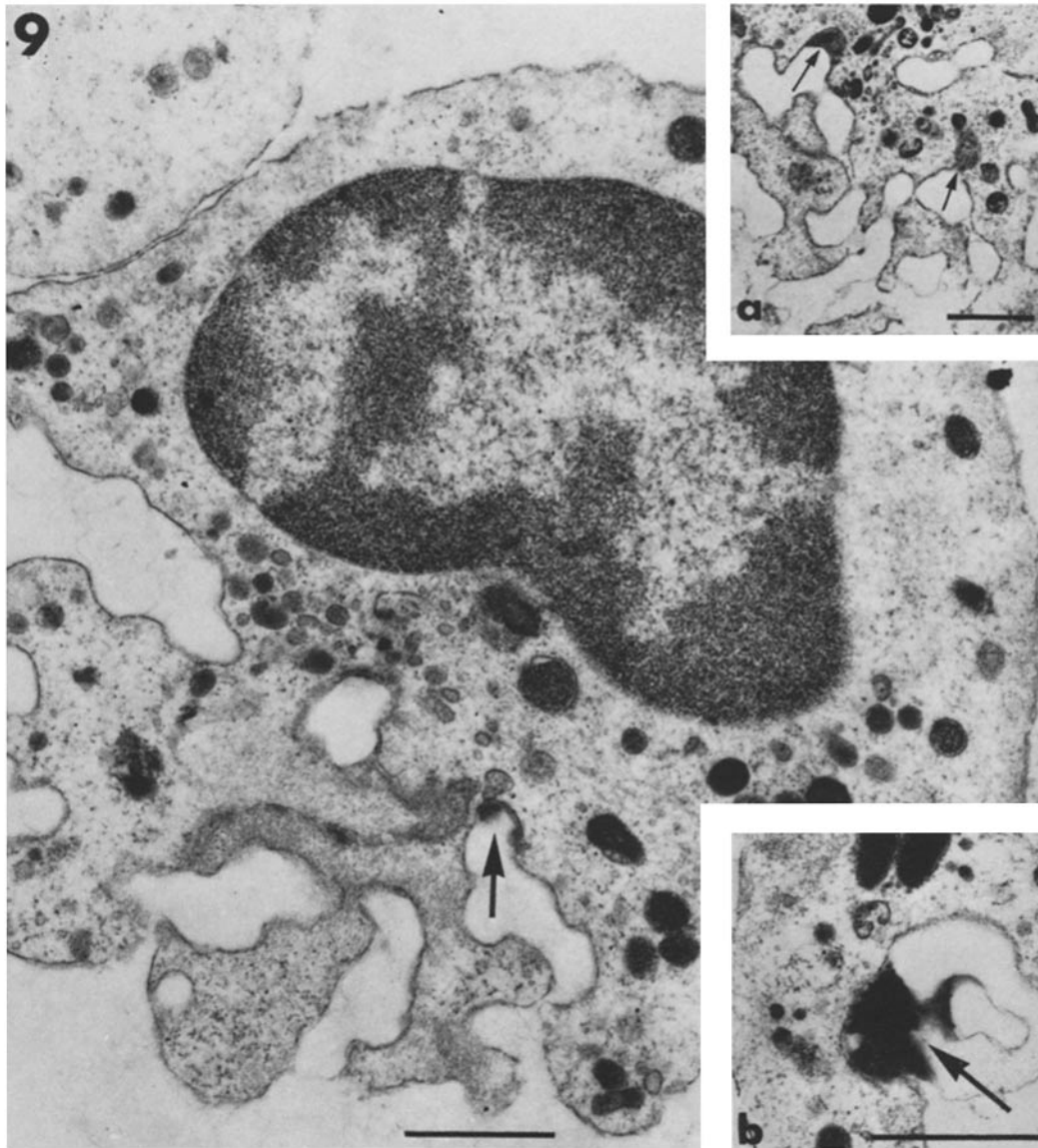
FIGURE 9 Human neutrophil in a chemical gradient of chemoattractant (Gey's balanced salt solution upper compartment, N-f-Met-Leu-Phe [10^{-5} M] lower compartment) fixed in osmium-antimonate. At this concentration of synthetic peptide, pseudopods are observable although cells are not undergoing chemotaxis but rather are in the process of secretion. Examples of exocytosis (arrow and *insets*) can be seen at the side of the cell in contact with the micropore filter and closest to the chemoattractant. Uranyl acetate and lead citrate, × 20,000; *inset a*, × 11,800; *inset b*, × 23,000. Bar, 1.0 μm.

bound cations in human neutrophils are the nucleus, lysosomal granules, and the cytoplasmic membrane. Variation in the location of pyroantimonate deposits under different experimental conditions may reflect accumulation of cations or increased cation accessibility to antimonate. The

cation deposits may contain calcium as they were removed by EDTA and EGTA, and microprobe analysis indicated the possible presence of calcium ions, whereas Mg^{++} and Na^+ were not detected.

When randomly migrating neutrophils were fixed directly in osmium-pyroantimonate, cation

precipitates were observed in the condensed chromatin of the nucleus. In contrast, under conditions of chemokinesis and chemotaxis, cation deposits were found commonly but not exclusively in the uncondensed chromatin of the nucleus. The meaning of this observation is uncertain but may relate to metallic cations associated with nucleoproteins (18, 19, 22, 28).

Perhaps the most interesting chemoattractant-induced change in cation localization was associated with the plasma membrane. Localization of cations to the inside of the plasma membrane was observed only in cells stimulated by a chemoattractant and undergoing either chemokinesis or chemotaxis. In the former case, cations bound randomly to the inside of the cell membrane without obvious orientation while in the latter case the precipitate was associated with the inside of the cell membrane of pseudopods at the leading edge of the cell. This localized concentration of cations to the front of the cell during chemotaxis was unrelated to cell adhesion to the filter because submembranous precipitates were not observed in cells undergoing spontaneous random migration which made contact with and occasionally sent pseudopods into the filter. The presence of cations beneath the cell membrane may explain the decrease in negative surface charge observed in suspensions of neutrophils exposed to a chemoattractant (12). Whether or not deposition of submembranous cations represent localization of calcium-binding proteins or phospholipids in the cell membrane (24) which may be required for calcium modulation of actin filament contraction (9), or else localization of cation pumps as described in developing fucoid eggs (23), will await additional study.

Of considerable interest is the fact that deposition of submembranous cation was dependent on critical concentrations of chemoattractant. For example, with high concentrations of N-f-Met-Leu-Phe (10^{-5} M), which caused secretion of intracellular granule contents and depression of chemotaxis, no submembranous cation deposits were observed even though the cells appeared oriented and had pseudopods extending into the filter. Although the same chemotactic stimulus can cause both secretion and chemotaxis, the number of receptors stimulated and the sequence of events after stimulation may be quite different (1, 30, 32). Further experiments defining the role of local submembranous cation deposition in the modulation of neutrophil chemotaxis and secretion are currently under study.

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