Degranulation, Membrane Addition, and Shape Change during Chemotactic Factor-induced Aggregation of Human Neutrophils

SYLVIA T. HOFFSTEIN, ROBERT S. FRIEDMAN, and GERALD WEISSMANN
Division of Rheumatology, Department of Medicine, New York University Medical Center, New York 10016. Dr. Hoffstein's present address is Smithkline & French Laboratories, Philadelphia, PA 19101.

ABSTRACT Neutrophils stimulated by the chemotactic factor formyl-methionyl-leucyl-phenylalanine (FMLP) undergo a transient change in surface properties that permits the cells to adhere more readily to surfaces and to each other. This transient change can be monitored by light scattering as stimulated neutrophils form aggregates while stirred in a platelet aggregometer. Maximum change in light scattering occurs within 1 min and correlates with an increase in the percentage of cells that are in aggregates of four or more cells and a decrease in the percentage of single cells. With time (3–5 min), small aggregates disappear and single cells reappear. The transient change in adhesiveness is accompanied by a persistent change in cell shape; the cells become polarized and protrude ruffles from one sector of the cell surface. During aggregation the cells adhere to one another with smooth sides together and ruffles pointed outward. During disaggregation the cells dissociate laterally with the simultaneous internalization of membrane in the region opposite the ruffles. Particle bound to the surface by charge (thorotrast, cationized ferritin) are concentrated and internalized in this region. The change in cell shape from round to ruffled occurs within seconds, suggesting that membrane is added to the cell surface from an intracellular store. We therefore quantified surface membrane by electron microscopy morphometry and measured a 25% increase within 10 s of adding FMLP. The source of new membrane appeared to be the specific granule membrane since the kinetics of granule discharge (between 30% and 50% of all release occurs in the first 10 s) correlate with the appearance of new membrane. Furthermore, the amount of membrane that appears at the cell surface at 10 s correlates with that lost from intracellular granules in that time. Chemotaxin-induced aggregation thus begins with granule discharge and membrane addition followed by protrusion of ruffles. Adherence is maximal at 60 s and the gradual loss of adhesiveness that follows is associated with uropod formation and enhanced endocytic activity.

As has been well documented, neutrophils, after appropriate stimulation, rapidly remodel their surfaces. They undergo shape changes (24, 31), protrude ruffles, flatten, and spread on substrates. These activities would seem to require more surface area than is available in a rounded, resting neutrophil, since the surface to volume ratio of a flat object with ruffles is greater than that of a round object with few surface productions. These changes in cell shape are so rapid that it appears likely that the new membrane which forms ruffles must arise from a preformed intracellular store.

One possible source of preformed membrane is the large population of secretory granules that these cells contain. Granules in neutrophils are of two basic types, azurophilic and specific. Azurophilic granules are homologous with lysosomes in most other cells but contain, in addition to the hydrolytic enzymes active at acid pH, neutral proteases and the microbicidal agents, lysozyme and myeloperoxidase. Specific granules are unique to neutrophils and are less well characterized. They also contain lysozyme as well as lactoferrin and vitamin B-12 binding protein. It has gradually become apparent that discharge of these two granule types is controlled differently. Several secretagogues or chemoattractants stimulate the release

THE JOURNAL OF CELL BIOLOGY · VOLUME 95 OCTOBER 1982 234-241

of specific granules without the release of azurophils (10, 30), but none are known that do the reverse.

Gallin and his coworkers (5, 29, 30) have pointed out that neutrophils release a small but significant portion of their content of specific but not azurophil granules whenever they adhere to a surface or move toward an inflammatory stimulus, through a filter in vitro or through tissue in vivo. These maneuvers have in common the requirement for a shape change.

Another prompt neutrophil response to surface stimulation is an increase in adhesiveness. Cells stimulated by chemoattractants adhere better to surfaces than cells that have not been stimulated (4, 6, 19, 23). One surface to which these cells stick upon stimulation is the surface of another neutrophil (6, 14, 16, 19), so that stimulus-induced adherence can be readily measured by the reduction of light scattering as stimulated neutrophils form aggregates when stirred in a platelet aggregometer. We, therefore, studied the relationships among aggregation, changes in shape of the neutrophil, addition of plasma membrane, and discharge of specific granules from cells exposed to a chemoattractant.

We used the aggregation assay as a test system because it provided us with the ability to monitor continuously a transient change in adhesiveness. We were able, therefore, to correlate this change with membrane addition and granule discharge in an isotropic cell suspension that is amenable to morphometric analysis. Our results indicate that neutrophils increase by 25% the amount of membrane exposed to the outside after stimulation with the chemotactic peptide *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) (10^{-7} M). This increase in cell surface occurs within 10 s of adding stimulus and corresponds to the disappearance of specific granules and their membranes from the cytoplasm. Protrusion of ruffles and cell-to-cell adhesion occurs later, being maximal at 60 s. The decrease in adhesiveness that follows is associated with enhanced endocytic activity, localized to one pole of the cell.

MATERIALS AND METHODS

Human venous blood was anticoagulated with citrate and neutrophils separated by sequential Hypaque-Ficol gradient centrifugation, dextran sedimentation, and hypotonic lysis of residual red blood cells as previously described (11). The resultant cell preparation contained 95–98% granulocytes. Cell suspensions containing 3×10^7 cells/ml in PBS with 0.6 mM Ca^{++} and 1 mM Mg^{++} were stirred in a platelet aggregometer for 2–3 min to establish a baseline. FMLP (10^{-7} M final) was then added and stirring continued for another 3–5 min. A dual channel aggregometer was used and, while a recording was being made from one cuvette, samples were taken from the duplicate cuvette at four time points, as indicated by arrows on Fig. 1: immediately before the stimulus was added, 10 s after stimulus addition, at the height of the aggregation (usually 40–60 s after stimulus addition), and again after disaggregation occurred (usually 3 min after stimulus addition).

The samples, 50 μl, were taken from the stirred cuvette and placed into fixative containing 2% glutaraldehyde and 0.025% $CaCl_2$ in 0.1 M cacodylate buffer, pH 7.4. Some samples were reacted for the azurophil granule enzyme myeloperoxidase by the method of Graham and Karnovsky (7) and then incubated with cationized ferritin (Miles Laboratories, Elkhart, IN) 0.01 mg/ml before postfixation in 2% OsO_4. Others were not reacted for peroxidase but were washed in buffer, postfixed in 2% OsO_4, and then allowed to settle onto polylysine-coated cover slips. These latter samples were dehydrated in ethanol, dried in a Denton Critical Point Dryer (Denton Vacuum Inc., Cherry Hill, NJ), and then examined in a Jeol JSM 35U scanning electron microscope (courtesy of the Osborne Laboratories, New York Aquarium).

In some experiments the aggregometer was not used at all, but cells were incubated with tracer, either cationized ferritin (Miles-Yeda) 20 μg/ml, or thorotrast (Fellow testagar 10%), in the presence or absence of stimulus.

Morphometry

Thin sections of neutrophil pellets were photographed in a systematic sampling pattern (upper left corner of each grid square) for morphometric analysis of surface to volume ratios. Since our analysis was based on a study of thin sections, it was necessary to label the membrane compartments so that an invagination of the plasmalemma could be distinguished from a vacuole. Cationized ferritin, added to the cell suspensions after the primary aldehyde fixation, permitted us to define these compartments. This particle binds to exposed cell membranes but does not enter cells. Thus membrane with bound ferritin was defined as plasmalemma. Membrane compartments from which ferritin was excluded were defined as intracellular granule or vacuolar membrane. The latter was distinguished from the former by electron translucency, inhomogeneity of content, or both.

Whole profiles of neutrophils with at least three nuclear lobes were also photographed to obtain ratios of peroxidase-positive to peroxidase-negative granules. The photographs were made at × 4,083 and were printed at × 12,249. In some cases the photographs were made at × 153.60 and printed at × 46,107. A square lattice with 17-mm spacing was superimposed on each print for lower magnification prints and a lattice with 5-mm spacing used for the higher magnification prints. The number of intersections of the horizontal lines with each membrane compartment was counted as was the number of points that fell upon cell profiles. The S/v ratios were calculated from the equation $S/v = 2(I)/Lt$ (28), where I equals the number of times a test line intersected a membrane of interest and Lt equals the total length of test line, calibrated in microns falling on neutrophil profiles. Lt was between 500 and 1,000 μm for each sample within each of four experiments. S/v was determined for each sample and the results presented as the mean S/v plus or minus the standard deviation. P values were determined using Student's t test.

Lysozyme Assay

Neutrophils were incubated with 10^{-7} FMLP for various times and then rapidly centrifuged through oil in a Beckman Microfuge (Beckman Instruments, Spinco Div., Palo Alto, CA). Lysozyme activity (EC 3.2.1.17) in the supernatant was determined by the rate of lysis of *Micrococcus lysodeikticus* (Sigma Chemical Co., St. Louis, MO). Enzyme release was expressed as the percent of total enzyme activity released from neutrophils by 0.2% Triton X-100 (Rohm and Hass, Philadelphia, PA).

RESULTS

The changes in light scattering that occur when neutrophils are stimulated by FMLP while being stirred in an aggregometer are shown in Fig. 1. The maximum decrease in light scattering occurs within 1 min of stimulus addition but follows a time lag of 10–12 s. After a brief interval, light scattering begins to increase again and levels off by ~3 min poststimulation. The new level may be the same as that of the original cell suspension or it may have some intermediate level suggestive of incomplete disaggregation. Light microscope observations of cells fixed at various time points in the aggregation cycle showed that aggregation, measured as a decrease in light scattering, correlates with an increase in the percentage of cells in clusters of four or more and a decrease in the percentage of free single cells (Table I). Disaggregation, measured as a subsequent increase in light scattering, represents the reappearance of single cells and the disappearance of aggregates in the 4–10 cell size category. The

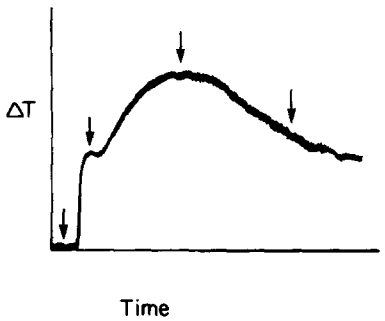


FIGURE 1 A typical aggregometer curve showing the changes in light transmittance (ΔT) that occur after addition of FMLP to human neutrophils (3×10^7/ml). The vertical arrows indicate the times at which cells were withdrawn for light and electron microscopy. The abrupt increase in transmittance between the first two arrows represents dilution of the cell suspension by 10% as FMLP was added to a final concentration of 10^{-7} M.

TABLE I

Change of Aggregate Size with Time during FMLP-induced Aggregation of Human Neutrophils

Aggregate size	% Cells ± SEM in each size category*			
	Control	10 s	Height of aggregation	Recovery
Single cells	84.6 ± 9.7	82.3 ± 7.4	17.0 ± 6.8	31.7 ± 12.4
2-3 cells	13.0 ± 9.3	12.3 ± 4.1	15.3 ± 2.2	12.7 ± 3.2
4-10 cells	2.3 ± 1.5	5.3 ± 3.4	36.0 ± 5.1	21.8 ± 5.1
>10 cells	0	0	28.7 ± 12.1	33.8 ± 17.8

* Neutrophils (3×10^{-7}/ml) were stirred in a platelet aggregometer and aliquots removed at the times and states of aggregation indicated. Cells were fixed in glutaraldehyde and examined by light microscopy. At least 200 consecutive cells were counted for each time point within each experiment, and the percentage of total cells in each aggregate size class was determined. The results presented are the means of three experiments.

coming apart of aggregates and the reappearance of single cells in the suspension can be masked by an increase in the size of the remaining clumps, which prevents the light scattering curve from returning completely to baseline. Whereas the mean size of clumps at the height of aggregation is 8.7 cells/clump and the median size is 7, during disaggregation the mean size of the remaining clumps is 16 and the median size is 11. In these preparations, some of the clumps contained 35 or more cells, but such superclumps were not seen at earlier time points.

Shape Changes

The transient change in neutrophil adhesiveness is accompanied by a persistent change in cell shape. Neutrophils, freshly isolated from peripheral blood, are rounded or slightly ruffled as seen by light microscopy. By scanning electron microscopy (SEM), the cells are seen to be not completely smooth but to have small ruffles and other surface irregularities (Fig. 2). Within 10 s of stimulation, the cells appear unchanged by light microscopy but by SEM an increase in surface detail is seen over most of the cell body. The increase is in the form of short ridges and folds suggestive of membrane redundancy (Fig. 3). By 30–45 s, the ruffles become fewer and longer and are predominately at one pole of the cell (Fig. 4). The remainder of the cell is rounded in contour with some small irregularities. By 3 min, the cells still maintain their ruffled polarized configuration but become elongated and smooth and form an antipodal tail or uropod, consisting of a cluster of small knobs at the narrowest point of the cell body (Fig. 5). Light microscopy of cells stained with Coomassie Blue indicated that >65% of stimulated cells have such tails, whereas only 15% or less of control cells do. The tails begin to appear after 90 s.

These tails consist of a cluster of cytoplasmic projections or blebs at the base of which are coated and uncoated vesicles. Coated vesicles are also prominent in adjacent areas but are not seen on the anterior two-thirds of the cell (Fig. 6; see also Fig. 10). The anterior of the cell bears evidence of this enhanced endocytic activity in the form of numerous vacuoles in the perinuclear cytoplasm. Bundles of 100-Å filaments are conspicuous in the posterior portion of the cell running from the nuclear lobes into the tail, and the vacuoles seem to be associated with these filaments (Fig. 7). All of these changes were seen in both stirred and unstirred suspensions and in cell suspensions too dilute to aggregate.

In stirred suspensions between 30 to 90 s after stimulation, the cells adhere closely to one another with their smooth sides together and their ruffles pointed outward (Fig. 8). These small aggregates are readily distinguished from the clumps that can form spontaneously in neutrophil preparations and also from the superclumps that sometimes formed later in the aggregometer. Whereas transient aggregates always have smooth sides together and ruffles extended outward, cells in randomly formed clumps have no preferred orientation and are frequently seen with ruffles touching. Observation with transmission electron microscopy at each of these time points confirmed the lateral association of adherent neutrophils with long stretches of parallel plasma membrane. Although the cells maintain large areas of contiguous plasma membrane, these never seem to touch, and cationized ferritin added after the cells are fixed is always able to penetrate these contiguous areas (Fig. 9).

During disaggregation, the cells elongate and part from one another laterally. Disaggregation is asynchronous within each clump (Fig. 10). It is associated temporally with a change in the distribution of surface change on each cell. Positively charged thorotrast particles or cationic ferritin added to cell suspension bind to the cells. In the absence of a chemotactic stimulus, the particles are distributed evenly over the cell surface and are endocytosed gradually in a nonpolarized fashion. The distribution of particles on the vacuole membrane in

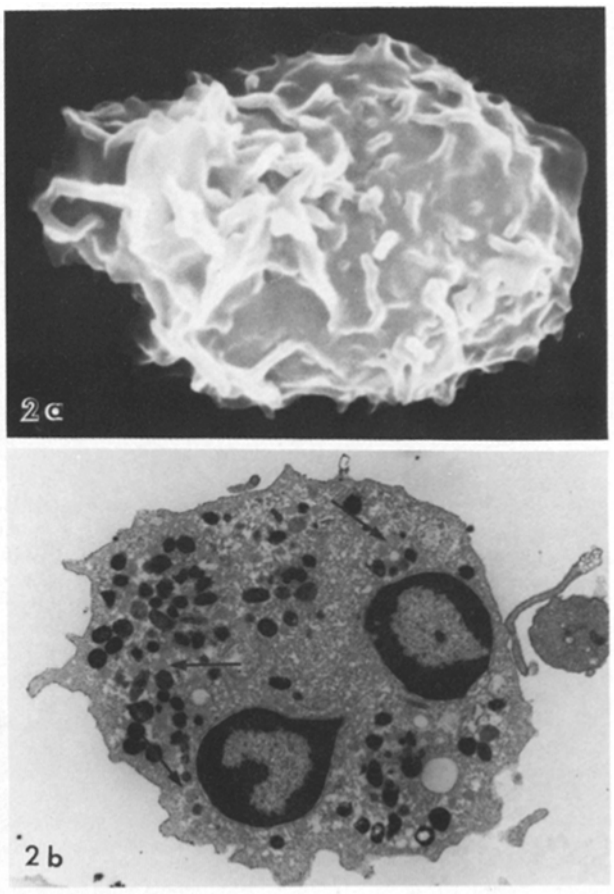
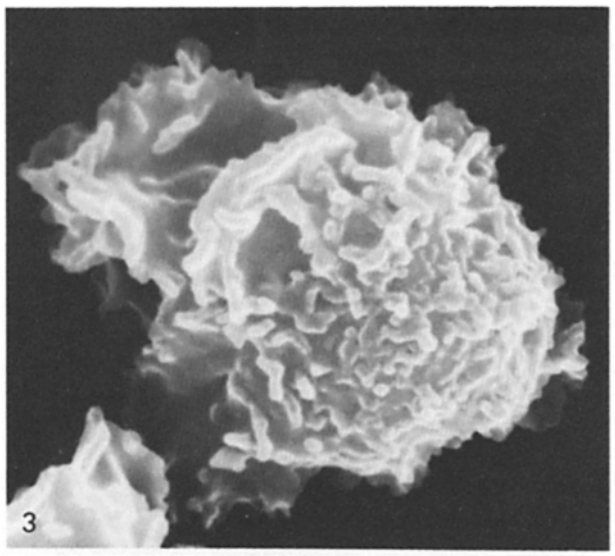
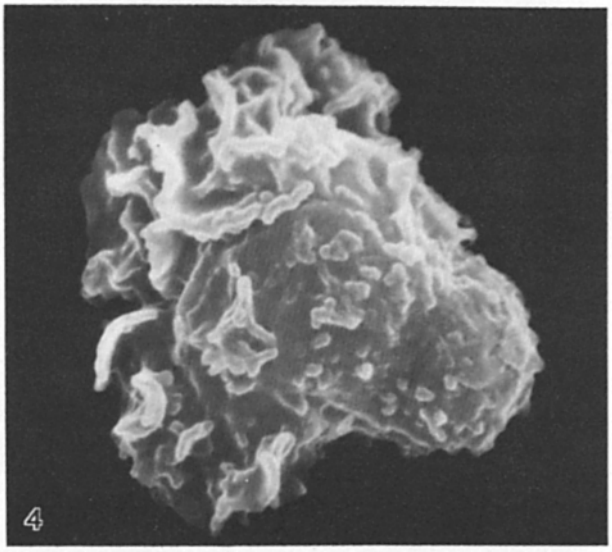


FIGURE 2 (*a*) A scanning electron micrograph SEM of a typical unstimulated neutrophil after Hypaque-Ficoll dextran sedimentation purification. After such purification procedures, neutrophils have a few short ruffles and membrane folds but the surface is smooth between these features. × 8,200. (*b*) A transmission electron micrograph (TEM) of a similar cell reacted for myeloperoxidase. Azurophil granules are peroxidase positive and appear black whereas the smaller, specific granules (arrows) are peroxidase negative and appear grey. × 7,800.





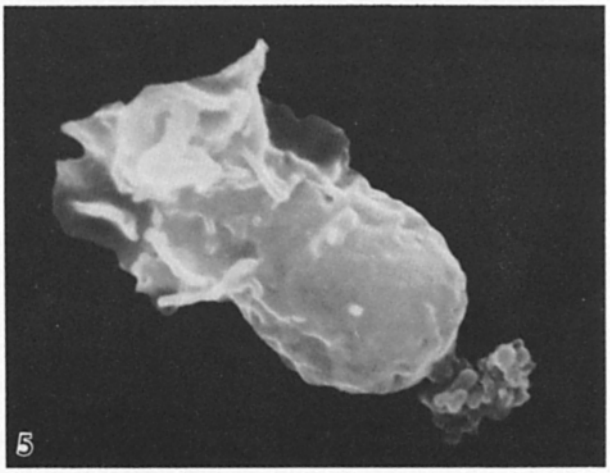


FIGURE 3–5 Fig. 3: An SEM of a neutrophil from the same cell preparation as that shown in Fig. 2 but in this case fixed within 10 s of FMLP addition. The short ruffles that were present before stimulation are still there and look as smooth as they did before stimulation, but a significant amount of new membrane has been added over the cell body, giving this region of the cell surface a rugged appearance. × 8,200. Fig. 4: An SEM of a neutrophil fixed 60 s after adding FMLP. The cells are now distinctly polarized with a tuft of ruffles at one end. The surface over the cell body is now smoother than at early time points. × 7,500. Fig. 5: After 3-min exposure to FMLP the cells retain their polarized tuft of ruffles but have formed in addition an antipodal tail consisting of a cluster of small cytoplasmic knobs. By this time the surface over the cell body has become even smoother, suggesting that a significant percentage of membrane is now in the tail. × 5,000.

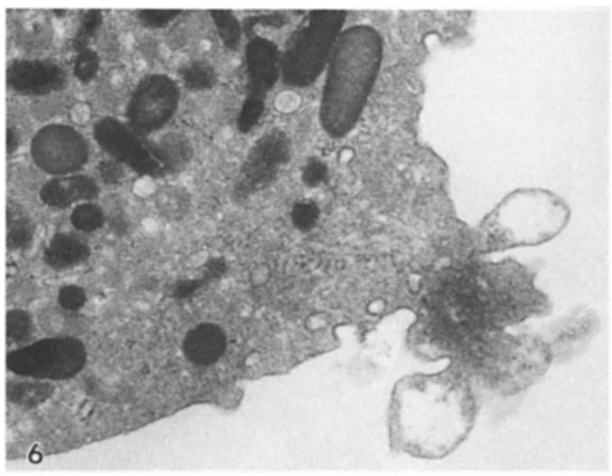


FIGURE 6 A TEM of a portion of a neutrophil 3 min after stimulation, showing coated pits being formed and internalized in the region adjacent to the tail. Little or no coated vesicle formation occurs anterior to this region. × 23,000

these cells is similar to that on the plasma membrane. However, when stimulus (FMLP) is added, the distribution of thorotrast becomes asymmetrical between 1 and 2 min. Particles are concentrated toward the rear of the cell and fewer persist on the ruffles. Vacuoles in the interior of stimulated cells have the same dense thorotrast distribution as the uropods, indicating that they form at that end of the cell after the distribution of particles becomes polarized (Fig. 11). Similar results are obtained with cationized ferritin.

Morphometry

The changes in cell shape observed during the aggregation cycle suggested to us that preformed membrane was added to the surface from an intracellular store. We, therefore, examined quantitatively, by stereologic analysis of electron micrographs, the addition of membrane to the neutrophil surface and found that after stimulation there was indeed an increase of ~25% in the amount of neutrophil surface membrane compared to unstimulated controls. Cationized ferritin was added to fixed cells and permitted us to distinguish exposed membrane from internalized membrane or vacuoles. As shown in Table II, the additional membrane is present as early as 10 s after stimulation and before either ruffling or aggregation is apparent by light microscopy. By this time, the surface to volume ratio changed from 1.26 ± 0.05 (control) to 1.57 ± 0.11 (stimulated) and remained essentially constant for the next 3 min. If we take the mean volume of the neutrophil to be 397 μm^3 (15), then resting neutrophils have 500 μm^2 of surface and stimulated cells have 623 μm^2; thus, 123 μm^2 of membrane are added to the cell within 10 s, an increase of 25%. In contrast, the 40–50 μm^2 increase in vacuole membrane shown in Table III becomes detectable only between 1 and 3 min.

Decrease in Intracellular Granule Numbers

Neutrophils stimulated by FMLP release lysozyme, an en-

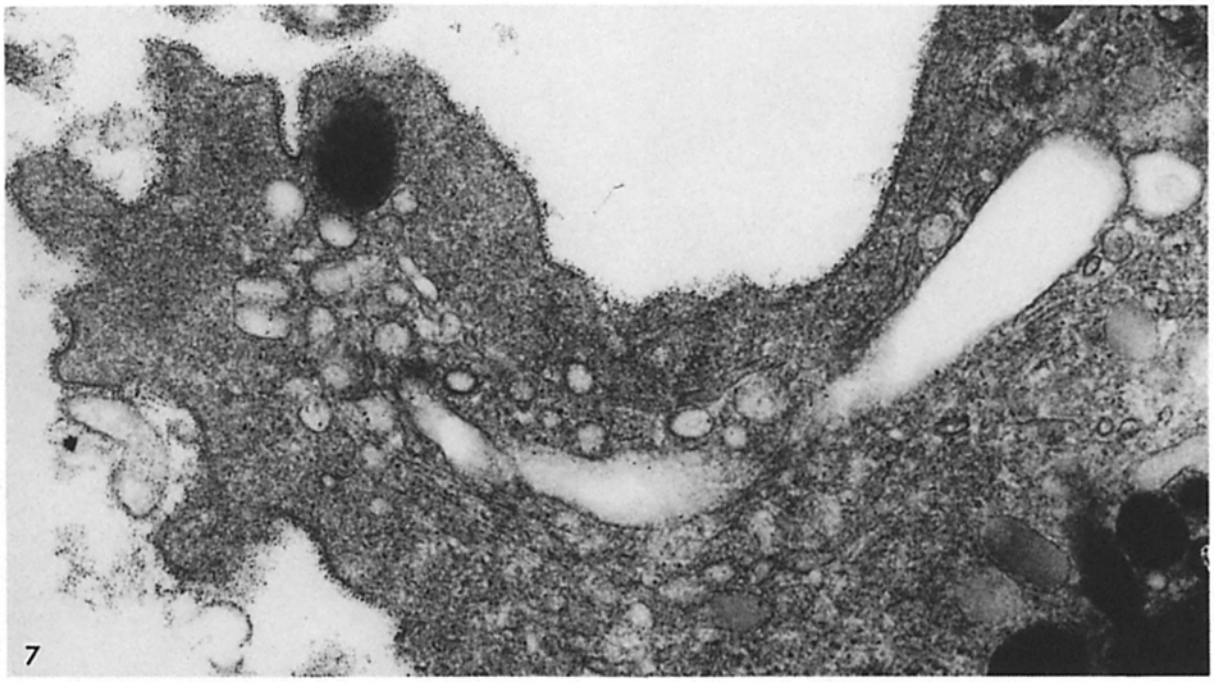


FIGURE 7 A higher magnification view of a section through the center of a tail showing that it contains both coated and uncoated vesicles and that the vesicles appear to exist in association with a bundle of 100-Å filaments that runs from the perinuclear cytoplasm into the tail. × 48,000.

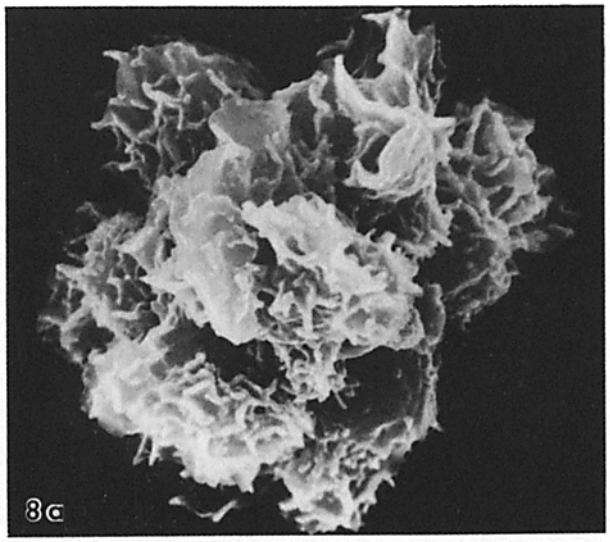
zyme distributed approximately evenly between specific and azurophil granules (3), but no β-glucuronidase, an enzyme present only in azurophil granules. We, therefore, reacted cells that had been fixed at various time points of the aggregation cycle for myeloperoxide (another azurophil granule enzyme [1]). Granule counts from electron micrographs of whole cell profiles (Table IV) show that the number of intracellular granules decreases abruptly within the first 10 s of adding stimulus and continues to decrease gradually thereafter. Separate counts of peroxidase-positive and -negative granules show that most of the change can be attributed to discharge of specific granules. Only a few percent of the peroxidase-positive granules are discharged. A degranulation event of that magnitude should theoretically lead to the appearance of ~20% of total lysozyme in the medium if each granule contained equal quantities of lysozyme. The release actually measured in 180 s is 12 ± 1% of total ($n = 6$). By 10 s, 34% ± 12% of that lysozyme was already free in the medium and by 60 seconds 82% ± 11% has been released ($n = 6$).

The limiting membrane of cytoplasmic granules was likewise quantified. As shown in Table V, granules in the resting neutrophil have approximately three times as much membrane as is present on the cell surface. After stimulation, the S_v changes from 3.71 ± 0.24 to 3.42 ± 0.07, a change of 0.49 representing a loss of 195 μm^2 ± 95 μm^2 of granule membrane. This amount of granule membrane is approximately equal to the amount that appears on the cell surface.

DISCUSSION

Much has been published in the recent literature suggesting that neutrophils stimulated by chemotactic factors are altered in many of their properties. Neutrophils thus stimulated discharge specific but not azurophil granules (5, 13, 16) and generate oxygen radicals at their surface (21, 22). Their net negative charge or zeta potential decreases (6, 8) and the cells are more adhesive to one another (6, 16, 19) and to endothelial cells (8) as well as to plastic- and protein-coated surfaces (4, 23). Stimulated cells are also reported to have a greater bactericidal capacity (12, 26) and to expose more chemotactic receptors (6). They also change shape and become more ruffled (24, 31). At the concentration of FMLP used in this study they become less deformable (14). The data presented here indicate that these reported changes in surface properties coincide with a 25% (≃125 μm^2) increment in plasma membrane area. This increase occurs so rapidly (<10 s) that the source of that additional membrane must be an intracellular, preformed membrane pool, probably the specific granule membrane. The data do not permit us to say whether the new surface membrane actually contributes to the observed changes in surface properties, especially adhesiveness, or whether granule discharge and increased cell adhesiveness are independent responses to chemotactic stimuli.

Most of the specific granule discharge induced by the chemotactic agent FMLP occurs very rapidly, with incorporation of the everted granule membrane into the plasma membrane, which increases significantly in area and develops ridges and folds. The bulk of the discharge and membrane addition occurs within 10 s of adding stimulus and precedes the protrusion of ruffles and increased adhesiveness that permits aggregation. Polarization in turn precedes the establishment of an organized uropod, since ruffles are formed and clustered at one end of the cell in less than 1 min and uropod formation occurs between one and three minutes' poststimulation. Aggregation *per se* does not appear to be necessary for any of the shape changes reported here since all stages occur at the same rate in conditions in which significant adherence is not observed, i.e., lower cell concentrations and unstirred cell suspensions. The elapsed time between stimulation and uropod formation with intensification of endocytic activity associate these phenomena with loss of adhesiveness in our experiments and with loss of receptors from the cell surface (25) and internalization of fluores-



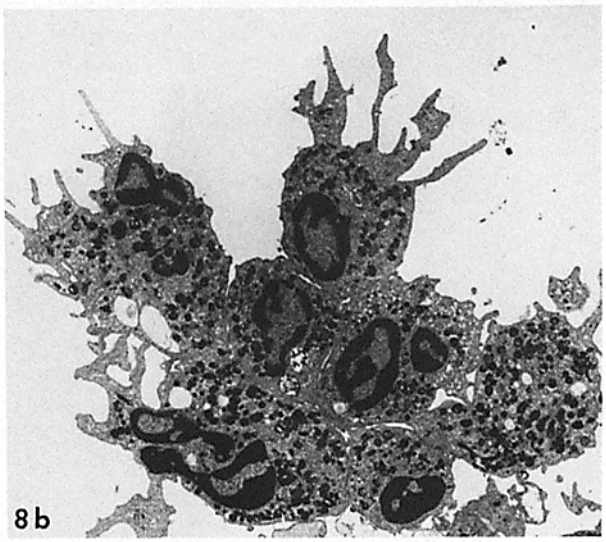
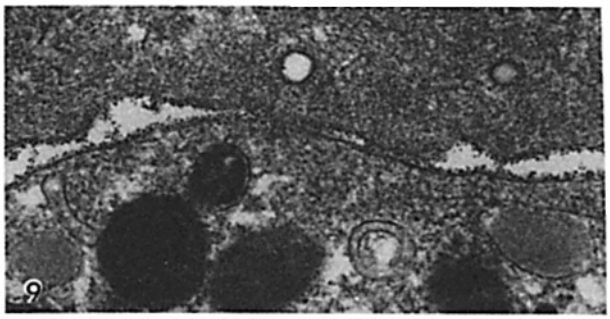


FIGURE 8 An SEM of a neutrophil aggregate. These cells were fixed 1 min after stimulation while being stirred in a platelet aggregometer. All of the cells have ruffles and in all cases the ruffles are pointing away from the center of the cluster. × 3,000. (*b*) A TEM of a neutrophil aggregate fixed as in Figure 8a and then reacted for myeloperoxidase. The cells adhere to one another by their smooth sides and their ruffles, containing few or no granules, project out into the medium. × 2,600.



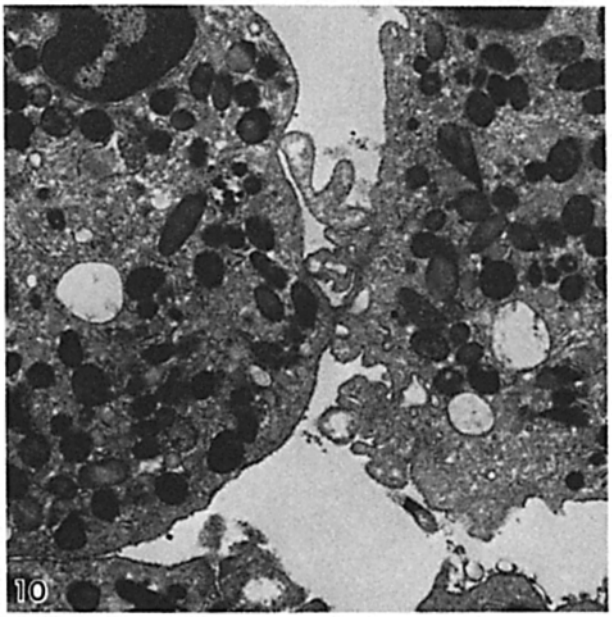


FIGURE 9 and 10 Fig. 9: A higher magnification view by TEM of the area between adjacent cells at the height of aggregation. Cationized ferritin added after fixation was able to penetrate even those regions in which the two plasma membranes were closest to one another, indicating that the cells approached one another no more closely than the combined width of their glycocalyces. × 53,000. Fig. 10: The center of an aggregate during disaggregation. The process is not synchronous in all cells of an aggregate. Thus, the cell at the upper right has separated from adjacent cells and is forming a tail, but the two cells at the left are still adherent to one another. × 6,000.

cently labeled ligand (17, 27), but we cannot yet say whether occupied receptor sites and adhesive sites are spatially related or whether these are internalized at the uropod.

Several possible sources of error are introduced when one attempts to calculate surface area from a surface to volume ratio. The results obtained are obviously contingent on the estimate of volume and assume that volume does not change significantly during the course of the experiment. We have shown (9, 10) by similar morphometric analysis that the fractional volume of all granules in resting neutrophils is 22.6% of the cytoplasmic volume (9) exclusive of nuclei, and that less than one-half of this volume is due to specific granules (10). Discharge of 40% of specific granules would therefore decrease cytoplasmic volume by <4.5% and have no effect on nuclear volume. The error this introduces into our calculation of S_v is therefore smaller than the standard deviation. Conversely, membrane internalization that accompanies the enhanced pinocytic activity of chemotactically stimulated neutrophils introduces a small but real increase in cell volume. These pinocytic vesicles are, however, quite small. Their volume as measured in our system was 1% of total cell volume before and immediately after stimulation and 3% 3 min later. Thus the loss of specific granules and the gain of pinocytic vesicles just about cancel out each other's effect on total cell volume and do not affect our results significantly.

A previous study has reported that chemotactically stimulated neutrophils increase in size (19). These studies used a Coulter type counter (Coulter Electronics Inc., Hialeah, FL) to estimate cell size. Since such systems measure size as electrical resistance and since the electrical resistance of a cell is a function of its surface membrane area (cytoplasm is conductive), formation of ruffles would be reported by such instruments as an increase in particle size. If the cells did increase in size by taking up water after stimulation, the denominator of our ratio would be larger than we assumed. However, the numerator or surface area would have had to be correspondingly larger since what was measured was the ratio of the two. Thus, if significant cell swelling did occur, we have understated

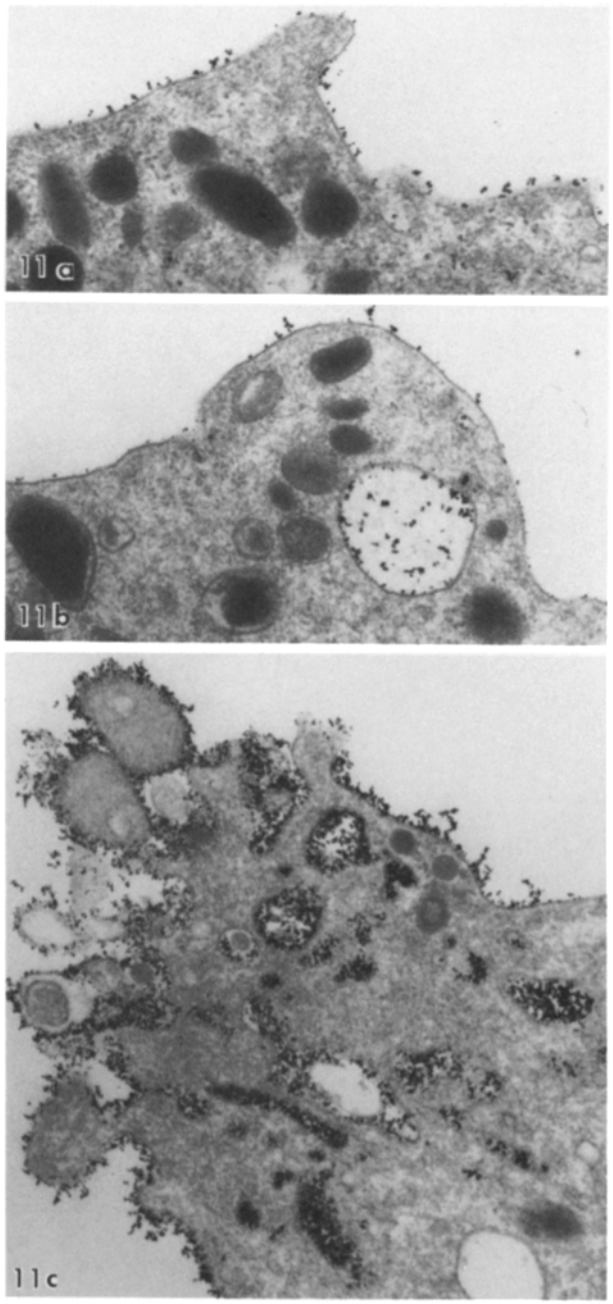


FIGURE 11 (*a*) A portion of a neutrophil that had been incubated with thorotrast for 2 min before fixation. The electron-opaque thorotrast particles are distributed evenly over the cell surface. (*b*) The corresponding region of a neutrophil incubated with thorotrast as in *a* but fixed 2 min after exposure to FMLP. The distribution of thorotrast in stimulated cells is much more uneven than in resting cells, and over most of the cell surface it is more meager. Its distribution in the vacuole shown in this figure is similar to that in unstimulated cells, indicating that the vacuole probably formed before the stimulus was added. (*c*) The uropod of the cell in *b* showing the accumulation and internalization of thorotrast in this region. These vacuoles are far more heavily labeled than that shown in *b*. × 26,500.

rather than overstated the extent of membrane addition.

The results of our morphometric analysis are similar to those obtained by Bowers et al. (2) who quantified surface in phagocytosing amoeba. They found that the plasma membrane area increased by 24% in phagocytosing cells, but that the cell surface to total volume ratio including phagosomes increased slightly, indicating that membrane was replaced on the surface during phagocytosis and that membrane intenalization and externalization were not tightly coupled. Their morphometric data suggested that in this cell small vesicles provide a pool of membrane that replaces surface membrane. Pryzwansky et al. (20) showed that PMN engaged in phagocytosing *Escherichia coli* discharged specific granules randomly over the cell body, suggesting that discharge of specifics may serve some function other than to contribute stored material to the phagosome content. This other function may be the same as that served by the small vesicles in amoeba, to replace membrane internalized during phagocytosis.

We do not yet know whether the membrane added to the surface by degranulation is necessary to confer special properties upon the cell such as increased adhesiveness or additional exposed receptors, but the increased surface membrane certainly contributes mechanically to the ability of the cell to perform various functions. For example, the ability to protrude ruffles and pseudopodia is helpful in migration and in contacting and internalizing bacteria. Furthermore, cells exposed to chemotactic factors continue to bind and internalize ligand into vesicles (18), taking from the surface a finite amount of membrane that increases with time. When rabbit PMN are chemotactically stimulated they increase their rate of fluid uptake from 1.7 to 6.6 nl/mins/10^7 cell and maintain this enhanced rate for at least 90 min (24). Similarly, membrane is removed from the surface by phagocytosis. As the surface area decreases as a result of either kind of stimulated endocytic activity, the surface to volume ratio would be expected to decrease and the cell shape become more spherical unless new membrane is added. It is our contention that the specific granule membrane is important in first achieving and then maintaining an appropriate surface to volume ratio for the

TABLE II

Increase in Surface Membrane during FMLP-induced Aggregation of Human Neutrophils

	S/v ratio*	±SD	μm^2/cell‡	±SD	*P* vs. control
Control	1.26	0.05	500	±20	
10 s	1.57	0.11	623	±44	<0.005
Height of aggregate	1.54	0.11	611	±44	<0.01
Recovery	1.66	0.11	659	±44	<0.005

* Calculated from the equation S/v = 2(I)/*Lt*. I equals the number of times a test line intersects neutrophil plasma membrane on an electron micrograph and *Lt* equals the total length of test line, calibrated in microns, falling on neutrophil profiles. *Lt* was between 500 and 1,000 μm for each time point within each experiment. $n = 4$.

‡ Calculated from S/v using 397 μm^3 as the mean neutrophil volume (determined by wet weight minus dry weight corrected for trapped water).

TABLE III

Increase in Intracellular Vacuole Membrane during FMLP-induced Aggregation of Human Neutrophils

	S/v ratio*	±SD	μm^2/cell‡	±SD	*P* vs. control
Control	0.09	±0.02	36	±4	
10 s	0.11	±0.02	43	±8	NS
Height of aggregate	0.10	±0.02	40	±8	NS
Recovery	0.24	±0.06	95	±23	<0.025

* Calculated from the equation S/v = 2(I)/*Lt*. I equals the number of times a test line intersects a vacuole membrane on an electron micrograph and *Lt* = the total length of test line, calibrated in microns, falling on neutrophil profiles.

‡ Calculated from S/v using 397 μm^3 as the mean neutrophil volume (determined by wet weight minus dry weight, corrected for trapped water).

TABLE IV

Decrease in Intracellular Granule Numbers during FMLP-induced Aggregation of Human Neutrophils*

	Granules/cell profile	% decrease	Azurophil granules/ cell profile	% decrease	Specific granules/ cell profile	% decrease
Control	159± 2.2		85 ± 1.7		74 ± 1.5	
10 s	133 ± 1.9	16.4	81 ± 1.5	4.7	52 ± 1.2	29.7
Height aggregation	130 ± 2.0	18.1	81 ± 1.6	4.7	49 ± 1.3	33.8
Recovery	127 ± 2.0	20.1	83 ± 1.6	2.3	44 ± 1.2	40.5
Cells with tails‡	116 ± 2.9	27.0	79 ± 2.4	7.1	37 ± 1.6	50.0

* Peroxidase-positive and -negative granules were counted from electron micrographs of neutrophils sectioned through at least three nuclear lobes. The data represent the mean number of granules per cell profile plus or minus the standard deviation of the counts from 31 or more micrographs for each time point ($n = 3$). A minimum of 4,000 granules was counted for each time point.

‡ Micrographs of cells in which tails were clearly visible were selected from the micrographs counted in the recovery and the granule counts from these cells are reported separately. These counts are from 14 micrographs; 1,627 granules were counted.

TABLE V

Decrease in Intracellular Granule Membrane during FMLP-induced Aggregation of Human Neutrophils

	S/v ratio* ± SD	N^2/cell‡ ± SD	*P* vs. Control
Control	3.91 ± 0.24	1,552 ± 95	
10 s	3.42 ± 0.09	1,358 ± 36	<0.05
Height of aggregation	3.13 ± 0.28	1,243 ± 111	<0.05
Recovery	3.22 ± 0.18	1,278 ± 71	<0.05

* Calculated from the equation $S/v = 2(I)/Lt$. I equals the number of times a test line intersects a granule profile on an electron micrograph and *Lt* equals the total length of test line, calibrated in microns, falling on neutrophil profiles. *Lt* was between 700 and 1,000 N. For each time point within each experiment. N equals 3.

‡ Calculated from S/v using, 397 μm^3 as the mean neutrophil volume (determined by wet weight minus dry weight corrected for trapped water).

function upon which the cell is engaged, replacing membrane lost to the interior by pinocytosis or phagocytosis.

We were aided by grants AM-25776 from the National Institutes of Health (NIH) to S. T. Hoffstein, and HL 19721, AM 11949, and AM 07176 from NIH to G. Weissmann. Dr. Hoffstein is the recipient of a Research Career Development Award (HL 00356).

Reprint requests should be addressed to Dr. Hoffstein.

Received for publication 14 January 1982, and in revised form 8 April 1982.

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