# Fc and C3bi Receptors and the Differentiation Antigen BH2-Ag Are Randomly Distributed in the Plasma Membrane of Locomoting Neutrophils

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*Abstract.* Reports from several laboratories suggest that neutrophils arrested during locomotion preferentially bind immune complexes at the front of the cell. Such assymetry of binding has been interpreted as indicating an active modulation of phagocytic receptors to the anterior of the cell. To investigate this further, we have used digital analysis of fluorescence images to determine the binding patterns of mAbs directed against the Fc receptors, the receptors for the C3bi fragment of C3, and a neutrophil-specific antigen. We found that all three proteins are distributed nearly identically along the length of migrating neutrophils, and their distribution very closely parallels the anterior to posterior distribution of the plasma membrane. The use of mAbs offered an important advantage in that the binding of antireceptor antibodies, unlike the binding of ligands, should be independent of potential changes in the affinity of the receptors. We conclude that the anterior distribution of the phagocytic receptors in the plasma membrane of locomoting neutrophils parallels the overall increase in membrane area at the front of a migrating cell and that specific translocation of phagocytic receptors does not occur.

DURING locomotion, the distribution of proteins on the cell surface results from a complex set of phenomena that may include bulk flow of the plasma membrane, rate of lateral diffusion, and the nature of the protein–membrane anchor as well as active redistribution of proteins based on the interaction with the cytoskeleton (14). Several investigators have attempted to elucidate the distribution of free (i.e., uncross-linked) neutrophil receptors on cells arrested in the polarized locomotor morphology. Observations with FITC–Con A, which binds to a wide range of mannosylated glycoproteins, have led to conflicting results. In one report, FITC–Con A binding was enhanced at the leading edge of locomoting neutrophils (32). Other investigators reported uniform binding of FITC–Con A over the entire cell (31). Receptors for the chemotactic peptide formyl methionylleucylphenylalanine (FMLP)[1] were found at the highest density at the anterior and the midbody regions of locomoting neutrophils (30). This distribution was irrespective of whether the cell was polarized in response to FMLP or to leukotriene $B_4$, indicating that the receptor distribution was a consequence of cell polarization and not of the occupation of the receptors by the ligand.

Two independent studies investigated the binding of opsonized sheep erythrocytes (E) to neutrophils that were first allowed to polarize and then were arrested by placement in an ice-cold buffer. The results showed that E opsonized with anti-E IgG [E(IgG)] bound to the front of 65% (34) or 98% (31) of polarized neutrophils. Similar observations were made with E opsonized with IgM and complement [E(IgM)C] (11). These particles were found to bind preferentially over the anterior region of the locomoting neutrophils (34; Michl, J., and P. Wilkinson, unpublished results). Several mechanisms for achieving this asymmetric binding are possible. First, frontal rosetting may result from the redistribution of the phagocytic receptors to the front of the cell. Unligated receptors are internalized at a constant rate by pinocytosis (20, 24). These receptors could move into the front of the cell as a result of the insertion of the membrane into this region (2, 5, 16). However, it is unclear how the Fc receptors (FcR) and complement receptors (CR) would be restricted from moving rearward with net plasma membrane flow. Another possible mechanism for frontal E(IgG) and E(IgM)C rosetting evokes a relative difference in affinity of the receptors located at the front as opposed to the rest of the polarized cell. Because the front of the cell is rich in cytoskeletal elements (23, 29), it is conceivable that phagocytic receptors in

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1. *Abbreviations used in this paper*: CR, complement receptor; FcR, Fc receptor; E, sheep erythrocytes; FITC-G anti-M Ig, IgG fraction of goat antiserum to mouse immunoglobulins derivatized with fluorescein isothiocyanate; FMLP, formylmethionylleucylphenylalanine; PMN, polymorphonuclear leukocyte; RT, room temperature; TMA-DPH, 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5,hexatriene, *p*-toluene sulfonate; TRITC-G anti-M IgM, IgG fraction of goat antiserum to mouse IgM derivatized with tetramethyl rhodamine.

this region are functionally more active. Little experimental evidence currently exists to support either of these interpretations. Finally, the anterior enhancement of immune complex binding may be a direct consequence of the forward redistribution of the plasma membrane. The ruffled front of a locomoting neutrophil most probably contains a greater surface area of the plasma membrane than any other cell region and, consequently, a higher net number of receptors. This membrane redistribution is evident in scanning electron micrographs of locomoting cells (1, 29), but has not as yet been quantitated.

In the present work, we attempted to determine whether specific phagocytic receptors are redistributed towards the front of locomoting neutrophils using several novel approaches: (*a*) mAbs directed against neutrophil phagocytic receptors were used in place of immune complexes to circumvent the problem of receptor affinity. (*b*) We measured the distribution of the plasma membrane of migrating cells using the fluorescent lipid probe 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5,hexatriene, *p*-toluene sulfonate (TMA-DPH) (15). The resulting fluorescence patterns were used to evaluate the contribution of cell morphology to the fluorescence images obtained with the mAbs. (*c*) The distribution of the membrane proteins was studied using the quantitative method of digital fluorescence image analysis. This allowed precise comparison of fluorescence patterns mediated by different mAbs on the same polarized and locomoting neutrophils and avoided the visual interpretation of images. (*d*) Finally, these experiments were made possible by the development of a novel cell migration chamber. We describe the preparation of this chamber and its use for the observation of neutrophil migration towards microscopic foci of complement activation.

Using these approaches, we were able to precisely quantitate the distribution of three plasma membrane antigens during locomotion, relative to the distribution of the plasma membrane. The plasma membrane antigens investigated in this study included the human neutrophil FcRIII, identified by the mAb 3G8 (12), CR3, identified by mAb IB4 (36), and the human neutrophil-specific antigen BH2-Ag, characterized with mAb BH2-C6 (26). The isotype difference between mAbs 3G8 and IB4 (murine IgG) and BH2-C6 (murine IgM) permitted an indirect double fluorescence study of two distinct antigens on the same polarized neutrophil.

We found that the anterior enhancement of receptor-specific fluorescence could be accounted for solely by the accumulation of the plasma membrane in the front of the cell. This finding strongly argues against active redistribution of receptors on locomoting neutrophils. However, as a consequence of membrane redistribution, the lamellipodium possesses a greater net number of receptors (not a greater receptor density) than other regions of the cell. Consequently, the lamellipodium may be the preferred region for the binding of ligands and ligand-bearing particles.

## Materials and Methods

### Preparation of Peripheral Blood Leukocytes

Neutrophils were isolated from peripheral blood of healthy volunteers by sequential dextran sedimentation (7) and density gradient centrifugation in Hypaque-Ficoll (Pharmacia Fine Chemicals, Piscataway, NJ) (3), as described in detail previously (26). Purified cells were washed by centrifugation in Hanks' basic salt solution (HBSS) and stored on ice. Viability was >97% by trypan blue exclusion.

### Preparation of Migration Chambers

35-mm tissue culture dishes (Costar Data Packaging Corp., Cambridge, MA) had a 1.2-cm hole punched out of the bottom. Coverslips (No. 1) were treated with chromerge, rinsed in water, air-dried, and attached beneath the holes with a 3:1 mixture of paraffin (Tissue-Tek, Fisher Scientific Co., Allied Corp., Pittsburgh, PA) and petroleum jelly (Vaseline) (17). The attached coverslips were treated for 30 min at room temperature (RT) with 5 μg/ml poly-L-lysine (PLL) (4,500–8,000 mol wt, Miles Laboratories Inc., Naperville, IL), rinsed with water, reacted for 30 min at RT with 1% glutaraldehyde and rinsed in PBS (21). A suspension of E was prepared at 0.001% hematocrit and washed four times by centrifugation in PBS. The chambers were filled with 4 ml of the E suspension and were incubated for 1 h at RT on a level surface. The chambers were rinsed vigorously with PBS and the attached E were fixed overnight in 0.5% formaldehyde in PBS. After fixation, the chambers were rinsed extensively in running water, incubated 1 h in room temperature in 0.1% lysine to block free aldehyde groups, rinsed in distilled water, and air-dried for storage.

### Neutrophil Polarization and Migration

The glass area of each chamber was incubated for 1 h at RT with a 100-μl rabbit IgM anti-sheep E (Cordis Laboratories Inc., Miami, FL) at four times the lowest subagglutinating dose (determined experimentally) and rinsed in PBS. Purified human neutrophils were suspended in HBSS to $5 \times 10^4$ cells/ml and 100 μl were added to each dish for 5 min at RT. Neutrophil migration towards the E targets was induced by adding 3 ml of 10% fresh human serum in HBSS at RT. After 5–10 min of cell migration, the chambers were rapidly emptied and flooded with ice-cold PBS containing 0.01% $NaN_3$. The cells were fixed in 1.25% glutaraldehyde for examination of morphology and in 4% paraformaldehyde for the indirect fluorescence assay (IFA).

### IFA of the Polarized Cells

The chambers were placed on a copper block cooled to 2°C (22). mAbs were added at saturating concentrations as follows: mAb 3G8 or IB4 (20 μg/ml in PBS with 1% heat-inactivated fetal bovine serum [HIFBS]) were added for 15 min, rinsed in ice-cold PBS, and fixed for 1 h in freshly prepared, ice-cold 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) in PBS. The cells were then sequentially stained at 5°C with affinity-purified, FITC-goat anti-mouse IgG (Organon Technika-Cappel, Malvern, PA) mAb BH2-C6 (15 min, 5 μg/ml) followed by affinity-purified rhodaminated goat anti-mouse IgM (TRITC-G anti-M IgM, μ chain specific; Organon Technika-Cappel). Second antibodies were added for 30 min at 2°C, at previously determined saturating concentrations. After each incubation, the cells were rinsed in ice-cold PBS. The cells were stored on ice until examined.

### Fluorescent Lipid Probe Staining

TMA-DPH (Molecular Probes Inc., Eugene, OR) was dissolved in dimethyl formamide to a stock concentration of 0.1 M (15). Migrating neutrophils were arrested by rinsing in ice-cold HBSS and stained with 5 μM TMA-DPH diluted in ice-cold HBSS. The cells were observed and videotaped at or below 4°C as described in the next section, using a 360-nm excitation filter.

### Quantitative Fluorescence Microscopy and Image Processing

Cells were observed using a Leitz Diavert inverted microscope (E. Leitz, Inc., Rockleigh, NJ) equipped with epifluorescence optics and a xenon lamp. Bright field and fluorescence images were recorded via a silicon intensification camera (SIT) on a videotape recorder and digitized using an image processor (IP 8500; Gould Inc., Imaging and Graphics Division, San Jose, CA) driven by a Microvax II computer (Digital Equipment Corp., Maynard, MA) (37, 38). Each digitized image had a 512 × 512 pixel format with an eight-bit linear-intensity range from 0 (black) to 255 (white). The black level and gain controls were manually adjusted for each image to optimize the intensity range while ensuring that no signal was lost at the low

end of the intensity scale. Linearity of the response for all components was verified as described previously (18). Dish background was established by measuring average intensity in four, 50 × 50 pixel fields that did not contain any cells, and was subtracted from all pixel intensity values.

The image was rotated so that the long axis of a polarized cell of interest was vertical. A rectangular area was defined that included the entire cell. The beginning and end of each cell was defined as the point at which average pixel intensity exceeded a threshold that was set at 1.5 × the intensity in adjacent cell-free areas. Measurements of fluorescence of cells stained with FITC- or TRITC-conjugated antibodies alone consistently gave maximum values below the threshold value for double-stained cells.

### *SWEEP Measurements*

The specific fluorescence of each cell was analyzed using a program called SWEEP. Within each horizontal line, the intensities of all pixels above the threshold were averaged. One such linear measurement is demonstrated in Fig. 4 *D*. The procedure was repeated for each horizontal line in the rectangle. The output of each SWEEP run consisted of pixel line numbers and corresponding average pixel intensities for each line.

Numerical data were converted into graphs of fluorescence intensity vs. distance from the front of cell. The results were converted into normalized fluorescence by assigning the value of 100 to the highest fluorescence obtained in a SWEEP run. The data for each cell were converted to bar graph form by dividing cell length into units of 10% and calculating average fluorescence intensities for all ten regions. The data were then pooled for all cells double stained with the same mAb combination.

## *Results*

### *Use of the Cell Migration Chamber*

The cell migration chamber used in these experiments is shown in Fig. 1. The rationale for its design was that opsonization of the E with IgM would lead, upon addition of fresh serum, to complement activation resulting in production and radial diffusion of C5a, the chemotactic fragment of C5 (27). The participation of complement in the stimulation of neutrophil locomotion in the chamber after a 10-min incubation is demonstrated in Fig. 2. In the presence of fresh serum, the majority of cells displays the distinct polar morphology of moving neutrophils (*A*). Furthermore, a large number of the cells (61%) have moved towards and over the opsonized E. By contrast, in the presence of heat-inactivated serum (*B*), the great majority of cells remains uniformly spread and only a few cells (7.5%) can be found over the erythrocytes. Fig. 3 shows the locomotion of neutrophils during a representative experiment. Tracings of cell positions were prepared from photographs taken at 0, 5, and 10 min after the addition of serum. In *A*, two neutrophils were observed in the presence of heat-inactivated serum. Although both cells initiated migration, the direction of locomotion did not ap-

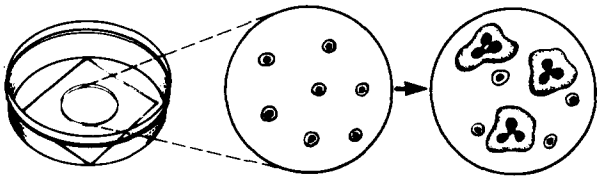

*Figure 1.* Diagram of the cell migration chamber. The preparation of the chamber is described in detail in Materials and Methods. The magnified region of the coverslip shows E covalently bound to the glass via a poly-L-lysine-glutaraldehyde bridge. The diagram on the right shows the appearance of a typical microscopic field before the addition of 10% serum.

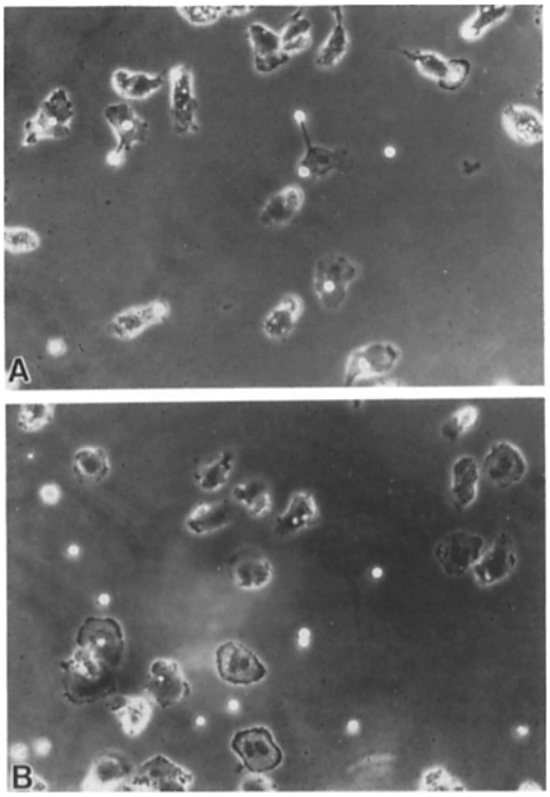


*Figure 2.* The effect of fresh and heat-inactivated serum on the behavior of the neutrophils in the migration chamber. The E were treated with R anti-E (IgM fraction) and 5 × $10^4$ polymorphonuclear leukocytes (PMNs) were added to each chamber. The chambers were flooded with HBSS containing 10% fresh or heat-inactivated serum and the cells were allowed to respond for 10 min at RT. The cells were fixed with 2% glutaraldehyde in PBS and photographed. (*A*) PMN responding to 10% fresh serum. Many cells have the typical polarized morphology of motile neutrophils. Several cells have moved over the E. (*B*) PMN responding to 10% heat-inactivated serum. The majority of the PMN are spread, and the cells have not moved towards the E. Photographed with a 40× objective.

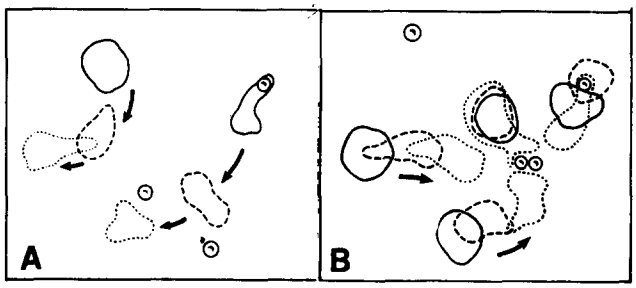


*Figure 3.* Motility of neutrophils towards erythrocyte targets. Neutrophils were allowed to adhere to coverslips containing IgM-opsonized E. HBSS containing 10% fresh or heat-inactivated serum was added and the behavior of the cells was photographed at fixed intervals using a 40× objective. Traces of the positions of the cells were made by projecting negatives onto paper with an enlarger. (*A*) Two cells migrating in the presence of heat-inactivated serum. The movement of the cells is not influenced by the presence of nearby E. (*B*) A group of neutrophils migrating towards a pair of E in HBSS containing fresh human serum. Cell positions: 0 min: ——; 5 min: - - - - -; 10 min: · · · · ·

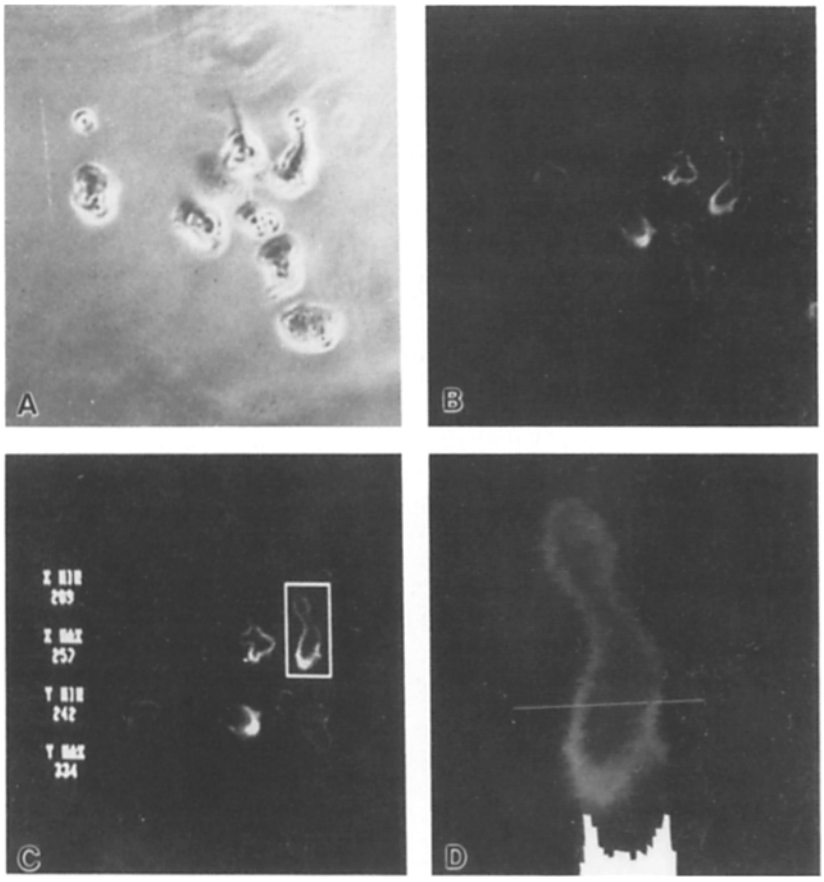


*Figure 4.* Digital processing of fluorescent images. (*A*) Phase–contrast photomicrograph of a group of motile cells. The motility tracings of the cells in this field can be seen in Fig. 3 *B*. (*B*) Fluorescent image of the cells stained with BH2-C6 and TRITC-G anti–M IgM. The photograph was taken from a computer screen after background subtraction. (*C*) A single polarized cell has been identified using coordinates indicated on the left of the screen. The image has been rotated counterclockwise by 15° to bring the long axis of the cell to the vertical. The rectangle shown consists of 4,416 pixel elements. (*D*) The image in *C* enlarged electronically four times. The pixel elements are visible. A white line crossing the plane of the cell indicates a sample measurement of a horizontal row of pixel intensities. A histogram of the intensity values along this line is shown below the cell.

pear to be influenced by the nearby E. A radically different neutrophil behavior was observed in the presence of fresh serum (*B*). Four neutrophils adhered to the coverslip about two to three cell body lengths from a pair of E. Upon addition of 10% fresh serum, all four cells initiated polarization and migration in the direction of the E.

These results demonstrate that a heat-labile signal (most likely C5a) is being released from the E. Neutrophils that move towards the E from a distance between one and five cell lengths maintain a distinct polarized morphology and direction of locomotion until reaching the target. The reproducibility of this observation permitted us to perform the studies described in the remainder of this paper.

### *Computer-assisted Measurements of Fluorescence*

The technique used for measuring fluorescence of individual cells is described in detail in Materials and Methods. A representative example of image digitization and processing is shown in Fig. 4. The directional motility of this group of cells has already been documented in Fig. 3 *B*. The cells were incubated with saturating levels of mAb BH2-C6 and TRITC-G anti–M IgM. A phase–contrast image of the cells at the moment at which their motion was arrested by flooding the dish with PBS at 2°C is shown in Fig. 4 *A*. The fluorescence images were videotaped and digitized. The appearance of the fluorescence following background subtraction is shown in Fig. 4 *B*. The rotation of the image and the definition of the region for SWEEP analysis are illustrated in Fig. 4 *C*. The SWEEP program repeats the linear fluorescence measurement described in Fig. 4 *D*, along the length of the cell. It is important to note that although the photograph (Fig. 4 *D*) shows little fluorescence along the middle of the scan line, the quantitative analysis, shown in the histogram in the bottom of 4 *D*, indicates that there is significant fluorescence in this region.

Graphic display of SWEEP analysis for the cell in Fig. 4 is shown in Fig. 5 *A*. *B* shows a conversion of intensity data into bar graph form. For this conversion, the average intensities of segments corresponding to 10% of the total cell length were determined. The objective of converting the data into bar graph form was to enable us to compare any one region of the cell with any other in a way that would be consistent from cell to cell. Since total cellular fluorescence varied from experiment to experiment and with the fluorophores used in double labeling, the measured fluorescence intensities were normalized to an arbitrary maximum value of 100 assigned to the highest segment average. This normalization is shown on the right hand ordinate in Fig. 5 *B*. It should be emphasized that the ratio of any two regions to one another is preserved in the normalization.

### *Comparison of the Distribution of BH2-Ag and the Phagocytic Receptors on Polarized Neutrophils*

These experiments took advantage of the difference in isotype of the mAbs BH2-C6 (IgM) and 3G8 (IgG). Polarized cells were incubated with mAb 3G8, fixed and sequentially

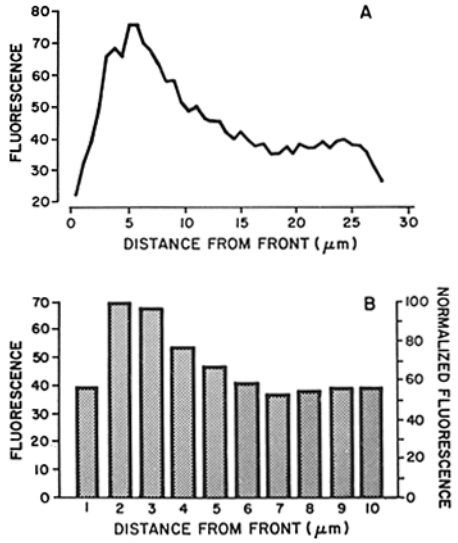


*Figure 5.* Graphic display and histogram transformation of SWEEP output. The fluorescent pattern of the neutrophil seen in Fig. 4 *D*, was analyzed using the SWEEP program. (*A*) Computer-generated graph of mean pixel fluorescence versus the distance from the front of the cell. Cell length was converted from pixels to microns using the value of 2.6 pixels per μm for an image digitized with a 40× lens. (*B*) Histogram transformation of data in *A*. The length of the cell (84 pixels) was divided into 10 regions of 8 pixels each with the final region consisting of 12 pixels. Cell region 1 is at the front of the cell. Mean fluorescence of each region was calculated and graphed in the form of a histogram. To obtain the normalized values shown on the right-hand ordinate, the maximum regional fluorescence value obtained (70, region 2) was assigned the value of 100 and the corresponding values of any other region were calculated by x = fluorescence/70 × 100.

incubated with FITC-G anti-M IgG, mAb BH2-C6, and TRITC-G anti-M IgM. FITC and TRITC images were digitized individually from each cell with preservation of spatial orientation. The fluorescence filters used prevented overlap between the fluorescein and rhodamine emissions.

Fig. 6 shows a representative migrating cell analyzed for the distribution of both BH2-Ag and FcRIII. The fluorescent images mediated by mAbs are shown in *A* and *B*. The peak of fluorescence intensity for each antibody (*arrowhead*) occurs in the same region of the cell with the maximum values for each separated by only 1 μm. Although the absolute intensities differ, the front to back distribution of fluorescence for each antibody is very similar.

The analysis shown in Fig. 6 was repeated with a number of cells double stained with the mAbs BH2-C6 and either 3G8 or IB4. The SWEEP data obtained with each antibody were normalized and means of fluorescence were calculated for 10 equal regions of cell length, as illustrated previously in Fig. 5 *B*. The normalization permitted the pooling of data from several cells double labeled with the same pair of mAbs. The resulting histograms of fluorescence distribution are shown in Fig. 7. *A* shows the distribution of mean normalized fluorescence on polarized cells double stained with mAbs BH2-C6 and 3G8. The distribution of fluorescence

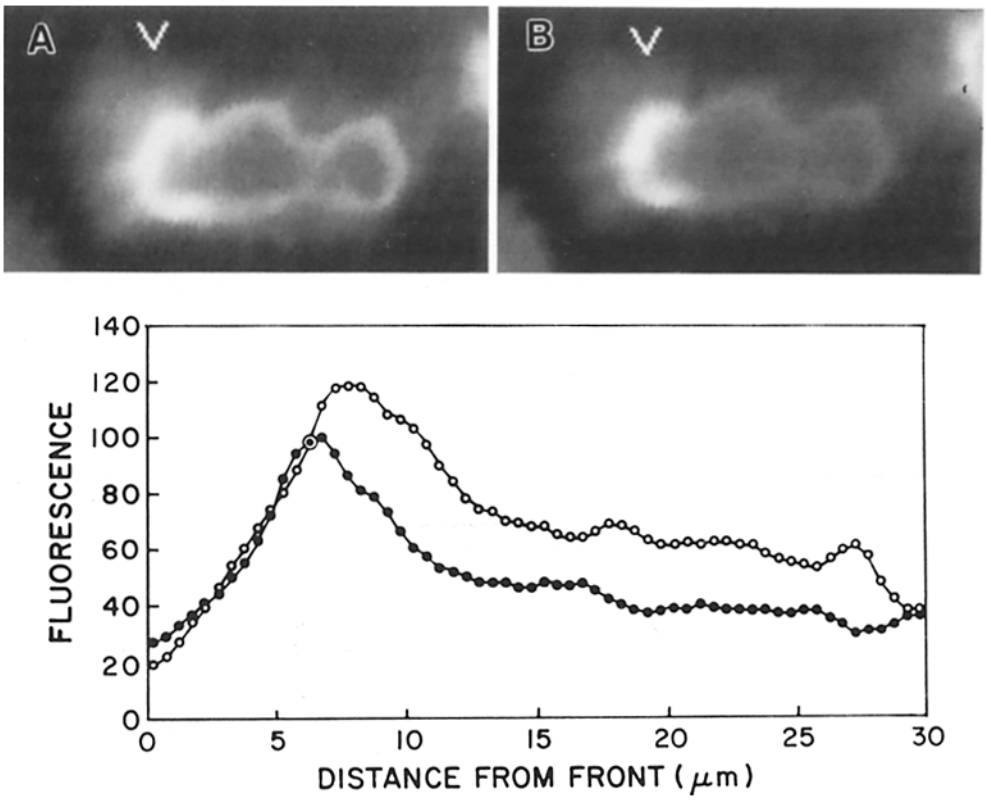


*Figure 6.* Computer-generated graph of fluorescence mediated by mAb BH2-C6 and 3G8 on a single polarized cell. Polarized neutrophils were reacted with mAb 3G8 and FITC-G anti-M IgG followed by mAb BH2-C6 and TRITC-G anti-M IgM. The fluorescence images were digitized and processed individually in a way that preserved the exact spatial orientation of the images relative to one another. (*A*) Photograph of BH2-C6-mediated fluorescence. (*B*) Photograph of 3G8-mediated fluorescence. The location of maximum average fluorescence obtained by SWEEP is indicated by an arrowhead. SWEEP analysis of BH2-C6-mediated fluorescence (○). SWEEP analysis of 3G8-mediated fluorescence (●). Cell length was converted from pixels to microns using the value of 4 pixels per micron for an image digitized via a 63× lens. Both images were photographed using identical settings of the monitor, the camera and the enlarger.

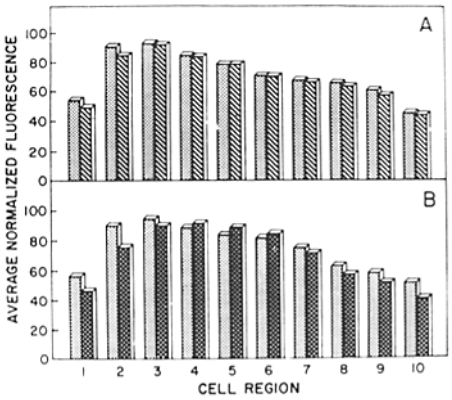


*Figure 7.* Bar graphs of mean normalized fluorescence on double-stained cells: Fluorescence distribution on polarized cells double stained with mAbs BH2-C6 and 3G8 (*A*; n = 20) or mAbs BH2-C6 and IB4 (*B*; n = 17) was obtained by SWEEP analysis of digitized images. The mean normalized fluorescence was calculated for each cell region as shown in Fig. 5, and the data were pooled for all cells double stained with the same mAb combination. The first bar in each cell region indicates normalized fluorescence obtained with mAb BH2-C6. Cell region 1 is at the front of the cell.

mediated by these mAbs was statistically indistinguishable ($p > 0.05$) in all 10 regions of cell length. This result indicates that the BH2-Ag and the FcRIII codistribute on the plasma membrane of migrating cells.

Nearly identical results were obtained with polarized neutrophils double stained with mAbs BH2-C6 and IB4 (Fig. 7 *B*). Of the 10 regions of cell length, significant difference in mean normalized fluorescence ($p < 0.05$) mediated by the two antibodies were obtained only in region 2. This finding indicates that the distribution of the CR3 on migrating neutrophils is very similar to that of the BH2-Ag and, by correlation, of the FcRIII.

It is evident from Fig. 7 that the maximum difference in mean normalized fluorescence from one region of the cell to another is only about two-fold (regions 3 and 10). This finding was obtained with all three mAbs used. Thus, the difference in the density of the BH2-Ag, the FcRIII, and the CR3, from the front to the rear of a migrating neutrophil, does not exceed a factor of two. The quantitative data presented in Figs. 6 and 7 were obtained under conditions which produce linear response to input intensity (19). As already pointed out (Fig. 4 *D*), the photographic printing process can enhance contrast, which is responsible for the apparent large increase in brightness at the leading edge of cells (e.g., Fig. 6).

### *Contribution of the Morphology of Polarized Cells to the Fluorescent Image*

Locomoting neutrophils assume a characteristic shape with a ruffled lamellipodium at the front and a wedge-shaped cell body gradually narrowing towards the end of the uropod (1, 9, 33, 39, 40). This redistribution of the plasma membrane towards the anterior could result in an increase of fluorescence in this region of the cell even if the density of membrane antigens remains uniform. Thus, it was important to establish whether the twofold difference in mean fluorescence intensity observed with mAbs over the length of locomoting neutrophils reflects a gradient of antigen density (per unit of membrane area) or unequal distribution of the plasma membrane. To address this question, we obtained SWEEP measurements of polarized neutrophils stained with the lipid probe TMA-DPH, which becomes fluorescent upon intercalation into the phospholipid bilayer (15). SWEEP measurements of several polarized neutrophils stained with TMA-DPH demonstrate a significant anterior accumulation of the plasma membrane on the polarized cells (Fig. 8). The front to rear distribution of TMA-DPH fluorescence was virtually identical to that obtained with the mAbs (Figs. 6 and 7). This result strongly suggests that the asymmetric fluorescence pattern observed with the mAbs is solely a consequence of the polarized plasma membrane topology of migrating cells.

## *Discussion*

The work described in this paper was prompted by the reports that the binding of immunocomplexes, such as E(IgG) and E(IgM)C3b (31, 34) or aggregated IgG (9), is greater at the anterior than at the rear of locomoting neutrophils. Most commonly, these results were interpreted to indicate that during locomotion a gradient of receptor density develops in the plasma membrane of the neutrophil. However, this would require that a mechanism exists for establishing and maintaining a gradient since unligated FcR and CR are freely mobile in the plane of the plasma membrane (12, 13, 21, 35). In fact, the human neutrophil FcR recognized by the mAB 3G8 (FcRIII, CD 16) has been shown to be anchored to the plasma membrane via a phosphatidylinositol anchor (28). Thus, in the unligated state, the FcRIII should be uniformly distributed on the plasma membrane unless it is associated with another, as yet unknown, protein. Unconstrained lateral diffusion should result in identical distribution of freely mobile membrane proteins (4, 10, 25). As an alternative to an active maintenance of a concentration gradient of receptors, we reasoned that anterior enhancement of immune complex binding could occur in the absence of active receptor redistribution if: (*a*) extensive ruffling of the plasma membrane at the lamellipodium provided a greater surface area in the front of the cell and, thus, a greater net number of receptors

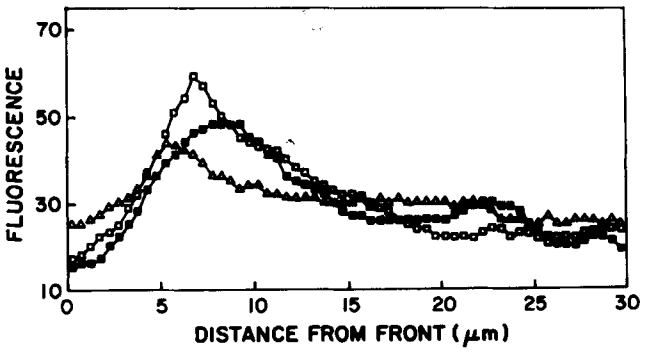


*Figure 8.* Distribution of the plasma membrane on polarized neutrophils. Background-subtracted images of polarized neutrophils stained with TMA-DPH were obtained and analyzed exactly as described for mAb-mediated fluorescence (Figs. 4–7). The graph represents SWEEP measurements of three individual migrating neutrophils. Pixel to micron conversion of cell length is the same as in Fig. 8.

(the density of receptors per unit membrane area remaining the same), or (*b*) the affinity of the receptors increased towards the front of the cell.

To determine whether phagocytic receptors are distributed towards the front of a moving neutrophil, we decided to use monoclonal antireceptor antibodies to localize these receptors on neutrophils arrested during chemotactic locomotion. This approach eliminates the possible effect of variable receptor affinity since it does not depend on ligand–receptor interaction. The binding of the mAbs was detected using two-color immunofluorescence analysis. We were aware in designing these experiments, that visual interpretations of fluorescence images have led, in the past, to contradictory results. For example, Weinbaum et al. (32) concluded from the appearance of fluorescence that receptors for ConA cluster in front of locomoting neutrophils. Using the same technique, Walter and co-workers (31) reported that these receptors exhibit uniform distribution. To circumvent this problem, we designed a quantitative image analysis method in which the fluorescence profile of a locomoting cell can be digitized and expressed as a function of cell length. This work was further aided by the development of a new neutrophil migration chamber that allows the observation of cell motility in a highly standardized manner (Fig. 1). Neutrophils exposed in this chamber to fresh serum take on the elongated, polarized morphology of migrating cells (41) and show directional movement towards the E(IgM) complexes (Fig. 2). This cell behavior is abolished by heat inactivation of serum (Figs. 2 and 3 *A*). These results strongly suggest that a soluble attractant, most probably C5a, is generated on the surface of the E (IgM) (6, 8, 27). Fig. 6 illustrates the difference between the impression gained from visual and quantitative analyses of fluorescence distribution. The visual appearance of fluorescence mediated by the mAbs BH2-C6 and 3G8 strongly suggests a significant redistribution of the BH2-Ag and the FcRIII to the front of the cell (Fig. 6, *A* and *B*). By contrast, quantitation of fluorescence over the entire length of the neutrophil (Fig. 6, graph) shows that significant densities of these proteins can be found in all regions of the locomoting cell. This result was generalized to a population of locomoting cells with respect to all three neutrophil plasma membrane proteins examined. As seen in Fig. 7, pooled values of normalized fluorescence mediated by the mAbs BH2-C6, 3G8, and IB4 are virtually identical in shape, indicating that BH2-Ag, FcRIII, and CR3 have nearly identical front-to-back distributions on a polarized neutrophil. The pooled fluorescence values show that the maximum measurable difference in fluorescence intensity and, consequently, the density of membrane proteins is only about twofold. However, parallel analysis of the distribution of the plasma membrane with the lipid dye TMA-DPH (15; Fig. 8) demonstrated distribution patterns nearly identical to those obtained with the mAbs. Thus, the front-to-back difference in intensity of mAb-mediated fluorescence can be accounted for entirely by the greater surface area of the plasma membrane in the anterior of locomoting neutrophils.

From these considerations, we conclude that the three antigens studied in the present work are distributed at uniform density per unit membrane area from front to back on the plasma membrane of locomoting neutrophils. This result is consistent with the current models of cell locomotion. Both the retrograde lipid flow model proposed by Bretscher (5) and the retraction-induced spreading model of Ishihara et al. (14) predict that the lateral motion of unanchored (unconstrained) membrane proteins should overcome the flow of bulk membrane lipid, resulting in random distribution. In fact, rapidly diffusing membrane proteins, such as Thy-1, remain evenly distributed during fibroblast locomotion (14). By contrast, the slowly diffusing fibroblast plasma membrane protein GP80 has been demonstrated to form a concentration gradient on motile cells, increasing towards the rear (14). This result cannot be compared directly with those obtained by us because of the difference in cell type used and because GP80, unlike FcR and CR3, does not undergo significant recycling.

In view of the results presented here, the asymmetric binding of opsonized E to polarized cells is not due to an active redistribution of phagocytic receptors. Furthermore, the frontal rosetting of E(IgG) is not due to the activity of the neutrophil FcRII (CD 32) since, in our hands, mAb 3G8 completely blocks E(IgG) binding to the neutrophils (26). The anterior binding of immune complexes must result from conditions that do not involve active movement of receptors towards the anterior. It is possible that the affinity of the phagocytic receptors in the front of a migrating neutrophil may be greater than at the cell body or the uropod. Since we did not use ligands to localize the Fc and C3bi receptors, our results do not address this possibility. However, to our knowledge, no experimental evidence for such an increase in receptor affinity is available. An alternative explanation, more consistent with the data presented in this paper, is that the frontal rosetting of opsonized E derives from the very topography of a motile neutrophil. Even though the receptor density per unit membrane area is uniform throughout the cell, the anterior region still possesses a greater net number of receptors as a consequence of membrane redistribution. Furthermore, the ruffled surface of the lamellipodium may allow for binding of several receptors to a spherical particle such as an erythrocyte, in effect increasing the avidity of the interaction. Thus, the shape of the migrating cell and the free diffusion of phagocytic receptors provide a simple mechanism for preferential phagocytic binding at the front of a migrating cell without requiring mechanisms for actively redistributing receptors.

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