BIOPHYSICAL ASPECTS OF MICROSPHERE ENGULFMENT BY HUMAN NEUTROPHILS

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ABSTRACT A quantitative investigation into the mechanism of neutrophil phagocytosis of opsonized microspheres possessing well defined dimensions was undertaken. Three aspects were documented: membrane conservation, cell adhesion to the spheres, and active cell cytoplasmic projection around the microspheres. The physical act of internalizing a particle by a cell involves a reduction in its plasma membrane area and an increase in its volume. As a consequence, a cell can internalize only a finite number of particles. A store of membrane area exists on cytoplasmic granules and may be recruited during phagocytosis. Previous measurements of neutrophil membrane area and volume served as a basis for estimates of the maximum number of internalized microspheres. A comparison with experimental prediction based on membrane conservation and degranulation agrees within 10% for a range of microsphere diameters, from 0.5 to 8 μm. This suggests that the limitation for additional particle uptake in the population of engorged neutrophils is the lack of excess plasma membrane area. In a random population of neutrophils, there was a sub-group, ~40%, which could no longer phagocytose before depleting their membrane stores. Several aspects of the engulfment process were investigated to elucidate the cause of this phagocytosis deficiency. It could be shown by single cell observation that these cases were associated with a lack of pseudopod projection, although adhesion was still evident between the cell membrane and the microspheres.

INTRODUCTION

Few cellular processes have received as much attention in recent years as the phagocytosis of particulate matter (Tsan and Berlin, 1971; Werb and Cohn, 1972; Stossel, 1973; Zurier et al., 1973; Leijh et al., 1979; Muller et al., 1980; Bowers et al., 1981; Besterman et al., 1982). Under in vitro conditions, granulocytes or monocytes will engulf a finite quantity of particles or microorganisms and then enter a period during which they are inactive (Werb and Cohn, 1972; Leijh et al., 1979). A phagocyte in this state will no longer take up additional particles and is stated to have reached its "phagocytic capacity." The number of microspheres, microorganisms, or immune complexes that a cell will engulf appears to be dependent on several aspects: the cell separation procedure, duration and type of opsonization, incubation temperature, method of cell/particle interaction, pH, and biochemical factors. The biochemical nature of the particle, its shape, size, electrical charge, and surface protein coat, also seem to affect phagocytosis. When these factors are optimized, phagocytosis should proceed until a cell has reached its phagocytic capacity. Three biophysical events are necessary for engulfment of a particle: adhesion between cell and particle, cell activation and projection of membrane-bound cytoplasm around the particle, and membrane fusion for enclosure. The adhesion and activation may involve specific adhesion membrane proteins and signal transduction at the site of particle attachment (Sklar et al., 1985; Boyles et al., 1981). Opsonization promotes phagocytosis of latex particles through deposition of labile serum factors on the particle surface (Hofacker and Wehinger, 1976).

Current evidence suggests that receptors mediating endocytosis can enter the cell, deliver a ligand, and within minutes be returned to the plasma membrane (Tsan and Berlin, 1971; Besterman et al., 1982). This possible shuttling of recycled receptors to the cell surface, along with the fact that cells at their phagocytic limit can often adhere to particles, would suggest that lack of receptors may not be the primary factor limiting phagocytosis. Phagocytosis is an energy-consuming process fueled by ATP, and a deficiency of ATP could stop phagocytosis (Hoffstein et al., 1974). Neutrophils are endowed with rich cytoplasmic stores of glycogen, providing the metabolic energy for pseudopod formation over many hours. The cells appear to phagocytose under aerobic and anaerobic conditions with equal efficiency, and inhibition of aerobic respiration may not limit phagocytosis. This is supported by the observation that most cells exhibit little effect on particle uptake in the absence of oxygen or in the presence of metabolic inhibitors (Karnovsky, 1962; Besterman et al., 1982). Phagocytic stimulation by polystyrene latex has been shown to be proportional to the amount of particles present. A concomitant increase in neutrophil metabolism is indicated by the increase in aerobic glycolysis and oxygen uptake for these stimulated cells (Roberts and Quastel, 1963).

Several investigators have suggested that one of the limiting factors in phagocytosis may be the availability of

membrane area. Human neutrophils are shown to have an excess of membrane area (Schmid-Schönbein et al., 1980) and in addition may recruit considerable amounts of membrane area from internal granules during phagocytosis (Hirsch and Cohn, 1960; Wright and Malawista, 1972; Hoffstein et al., 1974) or when stimulated with a chemotactic agent such as *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (Hoffstein et al., 1982). This process is termed "regurgitation during feeding" and is noted during phagocytosis of indigestable particles (Weissmann et al., 1971). However, no quantitative data are available for human neutrophils in support of these ideas.

The objectives of this study are to investigate membrane conservation during neutrophil phagocytosis of microspheres with a well defined geometry and to identify by direct measurement of cell–particle adhesion and observation of pseudopod projection the mechanism determining the extent of phagocytosis.

ANALYSIS

White blood cells in an isotonic medium have numerous surface folds which provide the cell with more membrane area than is needed to form a smooth sphere. In the case of human neutrophils, there is an excess of ~85% more plasma membrane area than is required to form a sphere of equal diameter (Schmid-Schönbein et al., 1980). An element of the neutrophil membrane surface is rather inextensible (Sung et al., 1982) and would rupture if the area is extended more than 2–3%. This property has also been found to apply to the membrane of the cytoplasmic granules, the mitochondria, and the nucleus (Schmid-Schönbein et al., 1980).

To determine how many microspheres, n, with volume V_{MS} and surface area S_{MS} can be internalized by a cell, we note that after internalization the initial cell volume, V_0, is increased by nV_{MS} and initial membrane area, S_0, is decreased by nS_{MS}. This assumes that the membrane of the phagocytic vacuole is closely apposed to the microsphere surface and that each sphere is taken up in a separate vacuole as is observed in most electron micrographs. After phagocytosis of n microspheres, the new cell volume, V, and cytoplasmic membrane area, S, are

$$V = V_0 + nV_{MS} \tag{1}$$

$$S = S_0 - nS_{MS}. \tag{2}$$

If, in the limit of complete plasma membrane engulfment, the cell becomes a sphere with smooth surface having radius, r, so that $V = (4/3)\pi r^3$ and $S = 4\pi r^2$, then by Eqs. 1 and 2 we find n in the form of a third-order polynomial:

$$n^3 a_3 + n^2 a_2 + n a_1 + a_0 = 0 \tag{3}$$

with the coefficients

$$a_0 = 36\pi V_0^2 - S_0^3$$

$$a_1 = 72\pi V_0 V_{MS} + 3 S_0^2 S_{MS}$$

$$a_2 = 36\pi V_{MS}^2 - 3 S_0 S_{MS}^2$$

$$a_3 = S_{MS}^3.$$

The solution to Eq. 3 yields two complex and one real value for n. For the case of interest here, the real value provides physical significance. Since S_0 and V_0 have been previously measured, a prediction of n for any size microsphere is possible (Fig. 1 *a*).

The experiments outlined below clearly show that Eq. 3

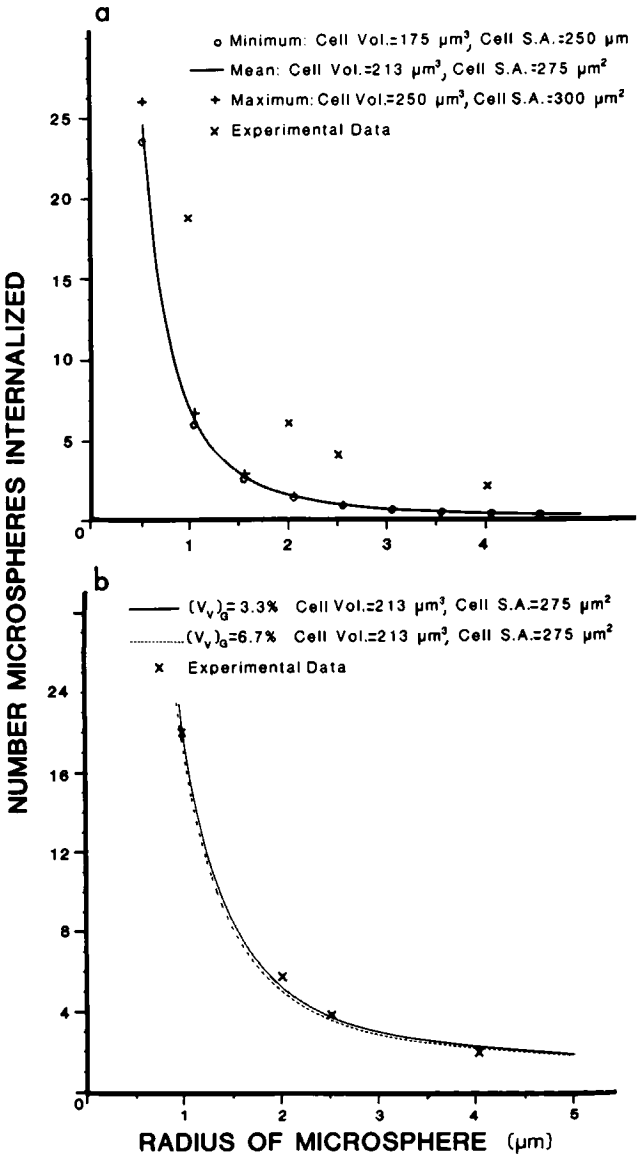

FIGURE 1 The maximum number of internalized microspheres by human neutrophils as a function of their diameter. (*a*) The theoretical prediction according to Eq. 3 without degranulation for three different cell volume and membrane area values as estimated previously (Schmid-Schönbein et al., 1980). The experimental values are shown by the crosses. (*b*) The theoretical prediction according to Eq. 6 with degranulation. Two cases of degranulation are shown, from a control granule volume ratio $(V_v)_G$ = 22.4% to $(V_v)_G$ = 3.3 and 6.7% as measured on micrographs after phagocytosis of 2.0- and 4.0-μm diam microspheres, respectively.

underestimates the actual number of microspheres that many neutrophils can engulf. This may be attributed to the neglect of the addition of internal membrane stores which may fuse to the plasma membrane. One possible source of such preformed membrane originates in the large number of secretory granules. It has been suggested that neutrophils selectively release specific non-azurophilic granules during stimulation by chemotactic peptides (Hoffstein et al., 1982) and during phagocytosis (Wright and Malawista, 1972). Stereology performed on nonphagocytosing neutrophils indicates that these cells carry between 3,000 and 6,000 granules (Schmid-Schönbein and Chien, 1988). During granule release, membrane is donated to the plasma membrane, and the cytoplasmic volume is decreased simultaneously. Under these conditions, Eqs. 1 and 2 become

$$V = V_0 + nV_{MS} - \Delta V_G \tag{4}$$

$$S = S_0 - nS_{MS} + \Delta S_G, \tag{5}$$

where ΔV_G is the total granule volume release and ΔS_G the source of additional membrane area. n can now be derived from a modified third-order polynomial:

$$n^3 b_3 + n^2 b_2 + n b_1 + b_0 = 0 \tag{6}$$

where

$$b_0 = 36\pi (V_0 - \Delta V_G)^2 - (S_0 + \Delta S_G)^3$$

$$b_1 = 72\pi (V_0 - \Delta V_G) V_{MS} + 3(S_0 + \Delta S_G)^2 S_{MS}$$

$$b_2 = 36\pi V_{MS}^2 - 3(S_0 + \Delta S_G) S_{MS}^2$$

$$b_3 = S_{MS}^3.$$

Eq. 6 leads to considerably higher estimates of internalized microspheres than predicted by Eq. 3. In the following experiments, a separate estimate of ΔS_G and ΔV_G will be obtained from electron micrographs, V_0 and S_0 will be taken from previous measurements (Schmid-Schönbein et al., 1980), and n computed from Eq. 6.

METHODS AND MATERIALS

Cell Separation

Fresh venous blood samples were obtained from several healthy human subjects by venipuncture in a heparinized vacutainer. The blood sample was transferred to a polypropylene tube, and the red blood cells were allowed to sediment at 37°C for 35–50 min. The supernatant plasma layer containing platelets, white blood cells (WBCs), and a few red blood cells were then pipetted into a second polypropylene tube. This separation procedure yeilded ~5 × 10^6 leukocytes: ~60% neutrophils and ~40% monocytes and lymphocytes. A few erythrocytes and platelets were also observed in the cell suspension.

Microsphere Phagocytosis and Single Cell Observation

Polystyrene latex microspheres (Dow Corning Corp., Midland, MI) were washed three times in phosphate-buffered saline (PBS) and then opsonized in 50% homologous plasma to PBS at 37°C with constant mixing for 15–25 min. Five sets of microspheres having diameters 0.527 ± 0.01 μm, 2.0 ± 0.01 μm, 4.0 ± 0.06 μm, 4.92 ± 0.09 μm, 8.0 ± 0.62 μm were used in this study. Their numbers were adjusted in each sample so that 5–10 times more microspheres were available per cell than their maximum predicted uptake.

The incubation mixture contained 0.5–1.0 ml of WBCs suspended in plasma to which an equal volume of PBS-glucose (10 mM) or Hepes-phosphate buffer was added. This mixture was incubated at 37°C in a slowly rotating water bath for up to 5 h. During this time, samples were taken for study under the light microscope.

At approximately ½ hour intervals, 25 μl of cells were suspended in a 50% plasma to PBS-glucose or Hepes buffer. This suspension was placed in a cell observation chamber maintained at 37°C and viewed through a 22-mm coverslip on an inverted microscope (Diavert; Leitz, Wetzlar, FRG) outfitted with a 63× oil immersion objective (NA 1.44) and a 25× eyepiece. A black and white video camera (IKC-40; Ikegami Tsushinki Co., Japan) was mounted on the mciroscope and a video cassette recorder (Panasonic AG-63000) was used to record the experiment for subsequent analysis. With this system continuous observation of single cells is possible. Two hydraulic micromanipulators (Narshinge Scientific Instruments, Tokyo, Japan) fitted with micropipettes were mounted to either side of the microscope stage. A pressure regulation system was connected to the wide end of each micropipette to facilitate cell aspiration at defined pressures. To prevent cell sticking and spreading on the coverglass or the micropipette, albumin (0.5 g%) was added to the cell observation chamber suspension media.

The Number of Phagocytosed Microspheres

One of the obstacles in phagocytosis research is that in many cases it is difficult to distinguish between a microsphere that is completely or only partially engulfed by the membrane projections. Thus, reliable determination of the number of particles incorporated into the cell's interior cannot easily be made. For example, even with high resolution light microscopy where the entire process of engulfment can be observed, it is frequently not possible to judge from a given number of microspheres associated with a cell, which are engulfed and which are merely attached. A precise determination of the number of intracellular microspheres is a prerequisite to a full analysis of the engulfment process. To circumvent this limitation and to obtain reliable numbers of fully engulfed microspheres, we used a technique of serial sectioning after fixation and embedding. On such serial sections, the degree to which a microsphere is engulfed by the cell cytoplasm can be determined without ambiguity and their numbers can be directly obtained. The area of membrane interiorized can then be estimated if each microsphere is enveloped in a discrete phagosome which is closely apposed to its surface.

For this purpose, cells that were allowed to phagocytose particles for ~2 h were fixed by addition of 2% glutaraldehyde in 305 mOsm cocadylate buffer (0.1 M cocadylate; Fisher Scientific Co., Pittsburgh, PA). The fixative was added at a rate of 1 drop/10 s with continuous stirring to bring the final glutaraldehyde concentration to 1%. After 1 h these cells were washed three times in cocadylate buffer, postfixed for 1 h in 1% osmium in cocadylate buffer (305 mOsm), washed in distilled water, and then dehydrated in ethanol diluted with distilled water in steps of 10% from 10 to 100%. The cells were rinsed in a 50% ethanol/acetone mixture followed by 100% acetone, infiltrated with 50% araldite (Polysciences, Inc., Warrington, PA) for 24 h, and finally embedded in 100% araldite and hardened at 60°C.

The tissue blocks were sectioned on an ultramicrotome (LKB Ultrotome V, Rockville, MD). Thick sections of ~1 μm were stained with toluidine blue (1%) and studied under a Leitz light microscope with a 63× objective (NA 1.44) and 25× eyepiece. Ultrathin sections ~300-Å thick were stained with 2% uranyl acetate (Polysciences, Inc.) and either Lead Reynold citrate (Eastman Kodak Co., Rochester, NY) or bismith nitrate (Polysciences, Inc.) and carried on a 200-mesh copper grid (Polysciences, Inc.). The sections were viewed on a transmission electron microscope (EM9S; Zeiss, Oberkochen, FRG).

For the case of the larger microspheres (4.0, 4.9, 8.0 μm), about 15 thick serial sections were prepared, and the same cells were identified and recorded in sequential sections, as shown in Fig. 2. This permits identification of each microsphere and recognition of the cell cytoplasm surrounding it. A clear classification of internalized versus merely adhering microspheres is then possible. For the case of smaller microspheres (0.5 and 2.0 μm), where large numbers may be present and direct counting is less reliable, a stereological procedure for electron micrographs was used as follows. Cross-sections from cells with phagocytosed microspheres and a relatively smooth cytoplasmic membrane (Fig. 3, *b* and *c*) were photographed at 1,600× and printed at 6,000×. The volume fraction of microspheres to total cell volume, $(V_v)_{MS}$, was determined with the point counting technique (Underwood, 1970). If V_0 is the initial volume of the cell (including nucleus and organelles) before phagocytosis, V_{MS} the microsphere volume, then the total volume of microspheres internalized is

$$nV_{MS} = (V_0 + nV_{MS})(V_v)_{MS}$$

and the number of microspheres is

$$n = V_0 (V_v)_{MS}/\{V_{MS}(1 - (V_v)_{MS})\}. \quad (7)$$

Degranulation

The number of granules released during phagocytosis was estimated from electron micrographs using the point counting procedure. Let $(V_v)_G$ be the volume ratio of granules to cell cytoplasm (excluding the microspheres and nucleus), and V_G the average granule volume. Then the number of granules present is

$$N_G = V_0 (V_v)_G/\{V_G [1 - (V_v)_G]\}, \quad (8)$$

analogous to Eq. 7.

Degranulation was quantified by determining the difference in intracellular granules between control neutrophils and those that have phagocytosed. The total granule volume released is

$$\Delta V_G = [N_G \text{ (control)} - N_G \text{ (phago.)}]\ V_G, \quad (9)$$

where N_G (control) and N_G (phago.) is the number of granules before and after phagocytosis, respectively. V_G is the average granule volume. If the granule membrane area is incorporated during exocytosis into the plasma membrane, then the total gain in membrane area is

$$\Delta S_G = [N_G \text{ (control)} - N_G \text{ (phago.)}]\ S_G, \quad (10)$$

where S_G is the average membrane area per granule. Granule volume ratios, $(V_v)_G$, were measured directly from electron micrographs. V_G and S_G were measured previously (Schmid-Schönbein and Chien, 1988).

The sections selected for degranulation counts included cells with anywhere from zero microspheres to the maximum number of microspheres that can be phagocytosed, including cross-sections with completely unfolded cytoplasmic membranes. This study was limited to microsphere diameters at 2.0 and 4.0 μm.

Measurement of Excess Membrane Area

To estimate the amount of excess surface area available after phagocytosis, a local cell region was aspirated into a micropipette. Under these conditions, the cell membrane inside and outside the pipette becomes unfolded and the aspiration pressure is balanced by the isotropic tension in the membrane (Schmid-Schönbein, 1986). The outside portion of the cell forms a sphere. The steady-state length by which the cell is deformed into the micropipette was then recorded. By assuming that the aspirated segment of the cell has a cylindrical geometry, the surface area is

$$S_{ASP} = 2\pi r_p \ell_p,$$

where ℓ_p is the length of the cell projecting into the micropipette and r_p the internal pipette radius.

The number of microspheres that could be accommodated by this surface was then calculated as

$$N = S_{ASP}/4\pi r_{MS}^2,$$

where r_{MS} is the microsphere radius. N was determined as a function of the number of intracellular microspheres, n.

Measurement of Pseudopod Length

Since phagocytosis requires the projection of a pseudopod around the microsphere, pseudopod histories were recorded for cells in free suspension and in contact with a microsphere. Individual cells containing 0–4 microspheres (4.9-μm diam) were recorded for several minutes while the

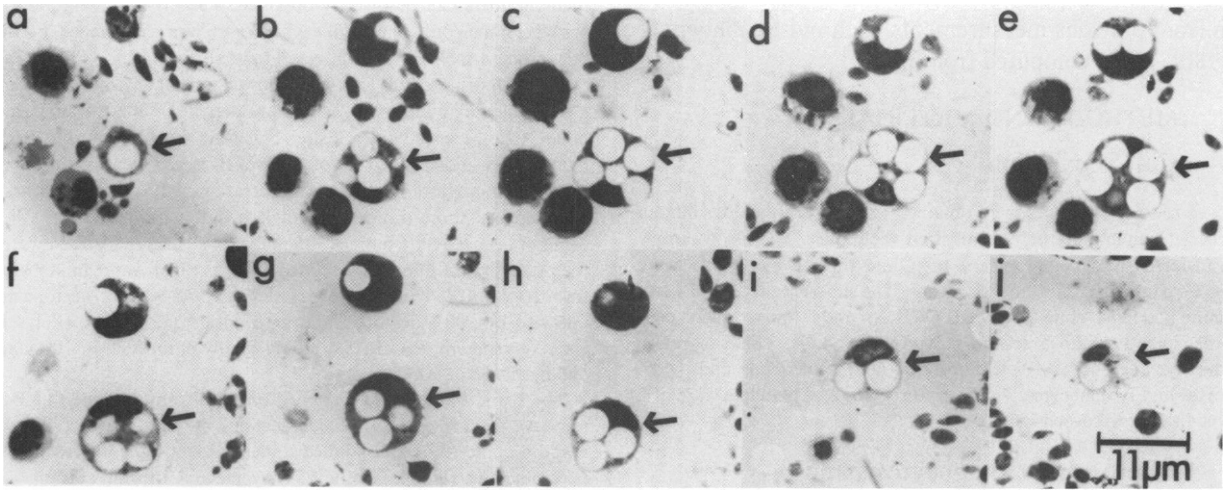

FIGURE 2 An example of the serial section technique to determine the number of intracellular microspheres. Each section is ~1-μm thick. The arrow in *a* points to the first of ten sequential sections passing through the cell. Five microspheres are evident through *d*, and a sixth microsphere comes into view in *e*.

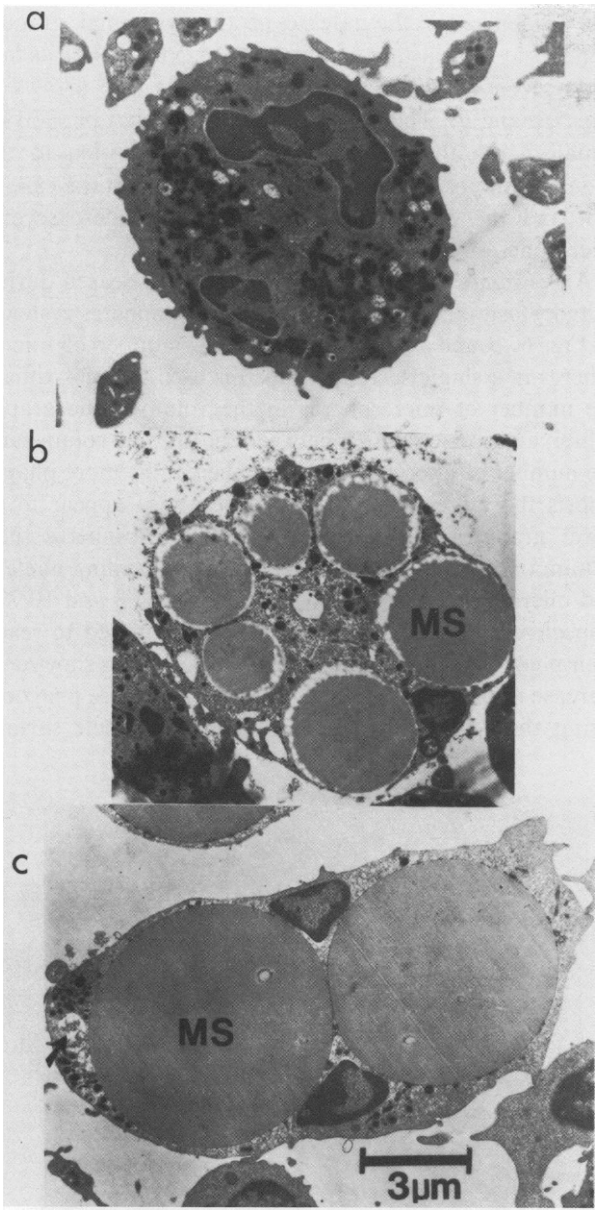

FIGURE 3 A control neutrophil is shown in *a*. Note the abundance of granules and excess membrane area in form of surface folds. A neutrophil with six 4.0-μm microspheres is shown in *b*. The microspheres are marked (*MS*). A neutrophil filled with two 8.0-μm microspheres is shown in *c*; there is almost no excess membrane area and granules are concentrated on the periphery. The arrow shows a region where degranulation has occurred in this cell. In *b*, the microspheres were partially dissolved during the embedding procedure leading to the appearance of partial shrinking.

cells were in free suspension without attachment to a substrate. During projection of single pseudopods, cells in free suspension frequently tend to rotate so that the newly projected pseudopod falls within the focal plane of the microscope. Subsequent analysis consisted of peak pseudopod length measurements by means of a video cursor (model 204A; IPM, San Diego, CA) controlled by a joystick. The positions were recorded by means of an A/D converter connected to a laboratory computer with appropriate length calibration (10 μm microscale; American Optics, Buffalo, NY) and video time record (IPM). The pseudopod length was normalized with the cell's resting diameter.

Microsphere–Cell Adhesion

To test the hypothesis that lack of adhesion between cell and microsphere may be the factor limiting a cell's phagocytic capacity, the adhesive stress between the phagocyte and the microsphere was determined by a dual micropipette technique (Sung et al., 1986). A neutrophil was held in place with one pipette while a microsphere was brought into contact with it via a second pipette. The degree of cell cytoplasm projection over the microsphere and the ensuing formation of a contact area was observed for a contact period of 5–30 s, at which time the microsphere was retracted. The microsphere–cell contact area, A_c, along with the pressure required to hold the microsphere and that required to hold the cell were recorded (Table II). The pressure in the holding micropipette was kept just high enough to anchor the cell without causing aspiration or cell lysis. The minimum pressure required in the microsphere holding pipette which could pull a tether of membrane from the cell without tearing the plasma membrane or dislodging the microsphere was designated as the critical adhesive pressure (P_c). The associated critical separation stress (σ_c) was calculated from P_c, the radius of the holding pipette, r_h, and the radius of the contact area between the cell and microsphere, r_c, as

$$\sigma_c = 2P_c\,(r_h/r_c)^2. \qquad (11)$$

σ_c was measured on 52 cells after phagocytosis of various numbers of the 4.9-μm diam microspheres.

RESULTS

Plasma Membrane Conservation

The predictions of the number of latex microspheres that a neutrophil can accommodate as a function of microsphere radius are shown in Fig. 1 *a*. The parametric curve is for a mean cell volume of 213 μm^3 and surface area of 275 μm^2. Also shown in Fig. 1 *a* are comparable numbers of internalized microspheres for a maximum cell volume of 250 μm^3 and surface area of 300 μm^2, and a minimum cell volume of 175 μm^3 and surface area of 250 μm^2. These values cover the full range of measurement errors for V_0 and S_0 (Schmid-Schönbein et al., 1980). Experimental mean values of the number of intracellular microspheres from a representative sample of neutrophils were derived from the histograms shown in Fig. 4. These mean values are plotted in Fig. 1, *a* and *b* and summarized in Table I. The experimental values of internalized microspheres between 2.0 and 8.0 μm are within 13% of those predicted by Eq. 6, but differ by 60% or more from the prediction provided by Eq. 3.

Degranulation

An abundant pool of membrane exists in the lysosomal granules distributed throughout the neutrophil volume (Fig. 3 *a*). Two cells that have reached their phagocytic limit with 4.0 and 8.0 μm microspheres are shown in Fig. 3, *b* and *c*, respectively. Comparison of the control cell and the cells at their phagocytic capacity shows an absence of surface folds and lack of excess plasma membrane. A decrease in density of the darkly stained cytoplasmic granules is also observed in cells at their phagocytic capacity (Fig. 3, *b* and *c*). Similar observations were made on neutrophils with other microsphere diameters. There

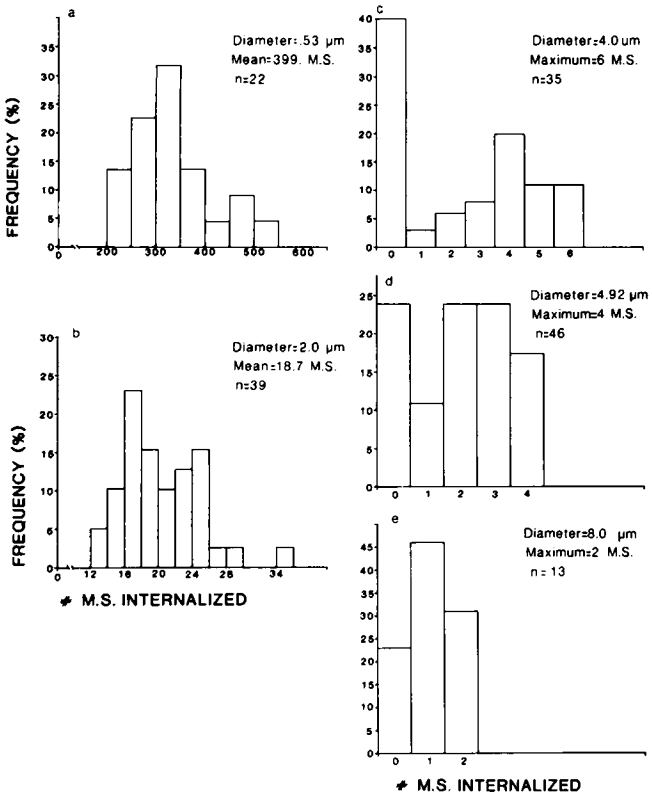

FIGURE 4 Frequency distribution for the number of microspheres internalized in a random population of human neutrophils after 5 h of incubation. The data in *a* and *b* were derived with the stereological technique (Eq. 7) and include only cell sections that exhibit at least one microsphere. The data in *c–e* were derived by serial sections and include a representative sample of neutrophils from these sections, including those without microspheres.

TABLE I
THE NUMBER OF MICROSPHERES INTERNALIZED BY NEUTROPHILS

Microsphere diameter	Theoretical prediction		Experimental measurement	Measurements by other investigators	
	Without degranulation	With degranulation			
µm					
0.529	114.1	704.0	399.4 ± 9.79*	360.0	(1)
2.0	6.9	19.2	18.7 ± 4.7‡	14.2 ± 2	(2)
4.0	1.4	5.4	6.0§	3.0	(1)
4.92	0.9	4.0	4.0§		
8.0	0.3	2.3	2.0§		

Theoretical calculations were for a mean cell volume, 213 μm^3 and surface area, 275 μm^2 and granule fraction $(V_v)_G$ = 3.3%. (1) Roberts et al. (1963). (2) Tsan et al. (1971).

*Mean value obtained by stereological technique on electron microscope.

‡Mean value obtained by stereological technique on thick sections.

§Mean value obtained by three-dimensional reconstruction of thick sections.

was evidence for the release of the lysosomal granule contents at the plasma membrane (Fig. 5 *b*), as well as into large secondary lysosomal compartments (see arrows in Fig. 5, *a* and *b*). The images show further that phagolysosomal membrane is usually in close apposition to the engulfed microsphere. Exceptions were seen in the case of 0.5- and the 2.0-µm diam microspheres where several microspheres often formed a single phagosome.

An estimate of the number of granules released during phagocytosis of the 2.0- and 4.0-µm microspheres is shown in Fig. 6, *a* and *b*. In this figure, the number of microspheres in a single section of a cell is used to approximate the number of microspheres in the volume. The graphs exhibit an inverse relation between the granule volume and the number of internalized microspheres; the more phagocytosis the more degranulation. There also appear to be fewer granules in cells with 2.0-µm microspheres than 4.0-µm ones, the granule to cytoplasm (excluding nucleus and microspheres) volume fractions being 4.8 and 10.7%, respectively. Neutrophils that had degranulated to reach their phagocytic limit with 2.0-µm microspheres showed an increase in their surface area by 237%, and those phagocytosing the 4.0-µm microspheres increased their surface

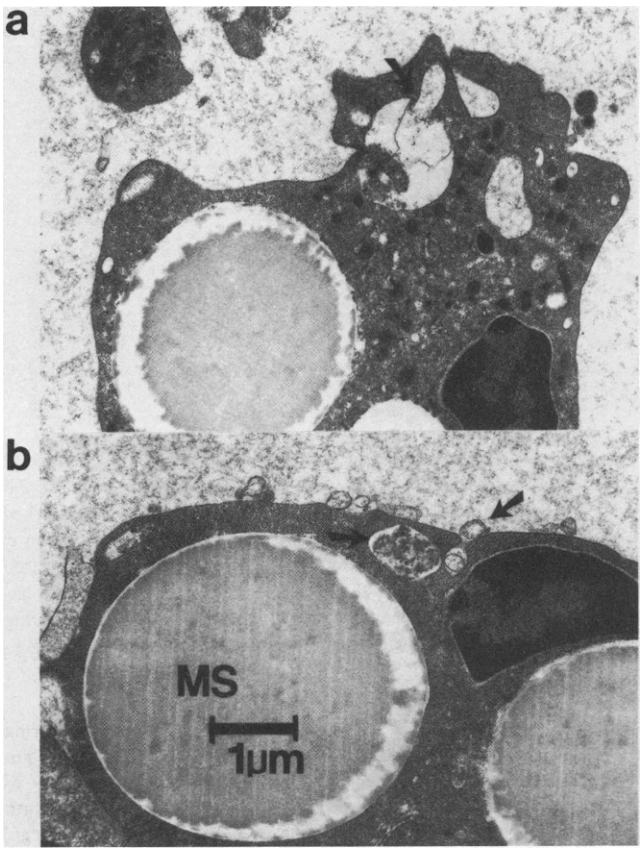

FIGURE 5 Electron micrograph of a secondary lysosome at the cell periphery (*a*, *arrow*). This lysosome appears to be in the process of extracellular degranulation. Primary lysosomes may also fuse with the plasma membrane and thereby release their contents extracellularly. A secondary lysosome (*arrow*) is also shown in *b*.

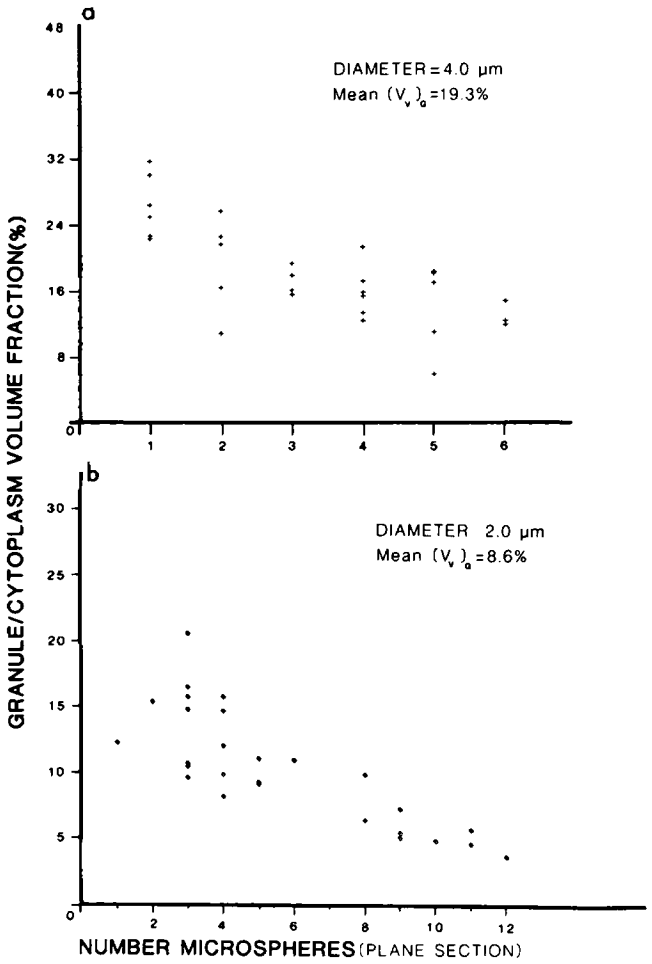

FIGURE 6 The volume fraction of granules and cell cytoplasm (excluding the nucleus) as a function of the number of internalized microspheres of two diameters (*a*) 4.0 μm, (*b*) 2.0 μm. Note that the number of internalized microspheres represents only a lower limit since these data were derived from a single electron microscopic section. There may be additional microspheres inside the cells out of the plane of section. The granule density can only be measured reliably on electron micrographs. The cells were fixed after 5 h of incubation when most had reached their phagocytic limit.

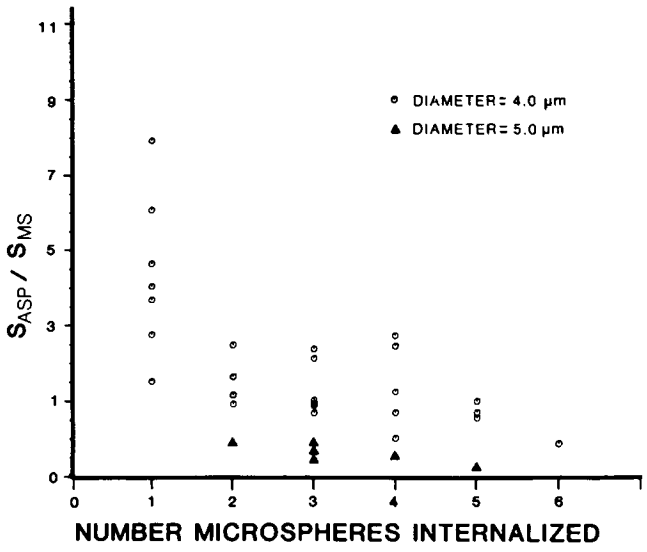

FIGURE 7 The excess plasma membrane in human neutrophils as a function of the microspheres internalized. The case for two different diameters is shown. The excess plasma membrane area was computed from the aspiration length ℓ_p and pipette radius r_p as $S_{ASP} = 2\pi r_p \ell_p$ and was normalized with the microsphere surface area S_{MS}. Note that the excess membrane area decreases during the progression of phagocytosis.

area by 202%. On the basis of these predictions, the revised number of phagocytosed microspheres was computed by Eq. 6, and the result is shown in Fig. 1 *b*.

Excess Membrane Area

Neutrophils that had engulfed latex microspheres were measured for excess plasma membrane by the micropipette aspiration procedure. Since excess membrane may be reduced during the process of engulfment, a cell that is filled with microspheres is close to its phagocytic capacity and should have few surface folds. The cell aspiration data support this principle of membrane conservation. Cells that contained fewer microspheres in their volume, as confirmed under the light microscope with micropipette manipulation, showed a larger aspirated lip and therefore more excess surface area (Fig. 7). Cells filled with either 4.0- or 5.0-μm spheres to their phagocytic limit were incapable of forming a tongue into the pipette. Furthermore, these cells would often lyse at a smaller aspiration area, S_{ASP}, than the unfilled cells. Fig. 7 shows the number of additional 4.0- and 5.0-μm microspheres the cell could accommodate with the excess membrane aspirated into the pipette. This was plotted as a function of the number of microspheres confirmed inside the cell volume. A clear inverse relationship is noted for both the 4.0- and 5.0 μm microspheres studied.

Pseudopod Time Histories

During incubation with the microspheres, many of the neutrophils in free suspension were observed extending and retracting pseudopods (Fig. 8). There was, however, a population of cells that were in a passive spherical state and did not form any pseudopods. Many of these cells were devoid of intracellular microspheres. Cells containing three or four microspheres showed smaller and less numerous pseudopod projections than cells with 0 or 1 microsphere (Fig. 8). The maximum pseudopod length in the latter is ~15% larger than in cells filled with between 2 and 4 microspheres.

When the neutrophils were brought in contact with the opsonized microspheres, a somewhat different picture arose. All neutrophils without microspheres, even those that were passive in free suspension, started to project cell cytoplasm around the microsphere, as is typically observed in phagocytosis. For cells that contained one microsphere, only 89% projected cell cytoplasm of those with 3–4 internalized microspheres, only 50% became activated

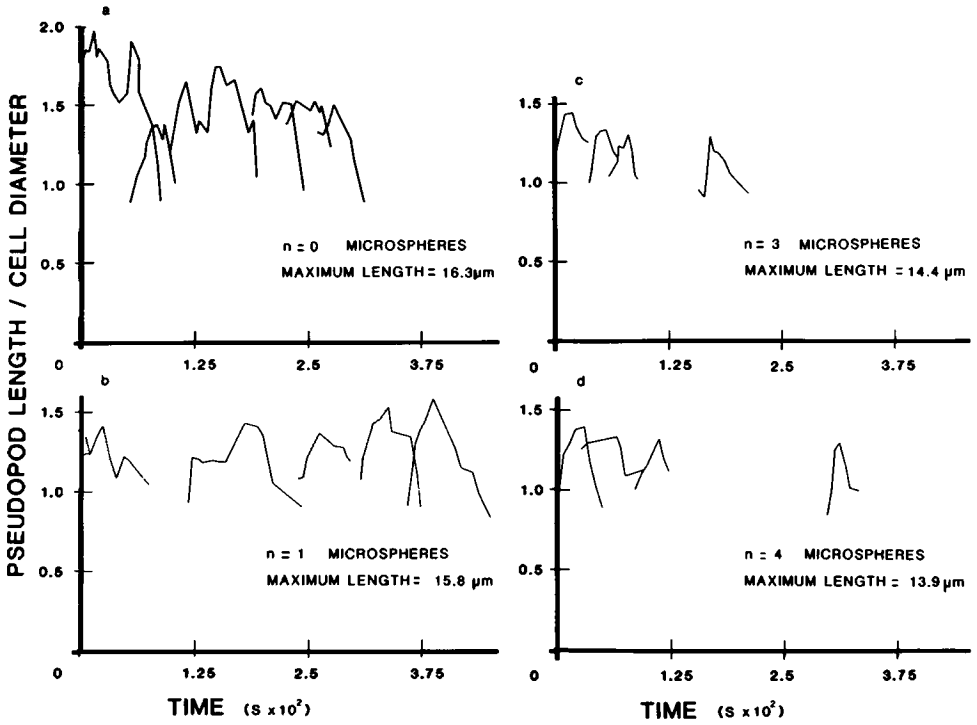

FIGURE 8 Pseudopod length in human neutrophils normalized with the cells undeformed diameter and plotted as a function of time. The major axis of cell deformation was measured as pseudopod length. Pseudopods often project from a deformed cell resulting in length–diameter ratios >1. The control cell in *a* demonstrates multiple pseudopod projects at a given instant. *c* and *d* show that filled cells send out fewer, smaller pseudopods with dormant periods in between. The maximum length of the pseudopods was measured as the average peak value over 5–10 pseudopod histories.

(Table II). This blunted response was seen even when the microsphere contact was maintained for prolonged periods.

Measurement of Adhesive Stress

The adhesive stress (σ_c) between the cell and microsphere was measured using one of two pipette pressures. It was sometimes observed that sphered cells filled with microspheres would not tolerate an appreciable amount of plasma membrane to be aspirated into the holding pipette. As a consequence, the cell attached to its micropipette was separated before the microsphere would detach from its pipette. In such cases, σ_c was estimated from the cell aspiration pressure since the net force between the cell and the microsphere had exceeded the force that held the cell to its holding pipette. The results are listed in Table II. In most cases a true critical separation stress could be measured, because the microspheres could be separated from the cell at their contact area.

The results show that the adhesive stress between microsphere and cell is evident both in cells close to their phagocytic capacity and in cells with abundant excess membrane. A comparison of the mean σ_c for 1 and 3 microsphere cells indicates that the 3 microsphere laden cells have an equal or greater amount of adhesive stress. Cells with fewer microspheres would form pseudopods and encapsulate the microsphere after contact more avidly than would filled cells. The number of cells that directed pseudopods toward and around the microsphere in response to a mechanical stimulation from the microsphere was greater for cells with fewer microspheres. Finally, the length of time necessary to form a conjugation area and a measurable adhesive stress was shorter for cells with 0 or 1 microsphere, ~5–10 s, than after engulfment of several microspheres, 20–30 s.

DISCUSSION

The current results support the concept that phagocytosis may be limited by the available membrane area and/or possibly by a lack of contact activation and subsequent pseudopod formation. Furthermore, in the case of freely suspended cells and microspheres in an incubation medium, the infrequency of encounter between cells and microspheres may represent a limitation. Only a small fraction of neutrophils showed a lack of contact activation, but all cells were subject to the membrane limitation.

The use of latex microspheres of known geometry enables one to estimate the area of surface membrane and volume internalized during phagocytosis. Since the particles cannot be digested, however, the state of phagocytic capacity may have to be distinguished from situations where the phagosomes contain digestible matter, as with bacteria. In the latter case, the phagosomal volume may shrink in time, and membrane material may be returned to the plasma membrane.

Three assumptions are made in the current analysis: (*a*) the cell does not swell, (*b*) internalized membrane is not shuttled back to the plasma membrane, and (*c*) new membrane is not produced by the cell during the time course of the incubation. (*a*) Since our analysis was based on a constant value of V_0 before and after phagocytosis and since the theoretical prediction closely approximates the

TABLE II
CRITICAL ADHESIVE STRESS AND PSEUDOPOD FORMATION MEASURED FOR NEUTROPHILS

Microspheres (d = 5 μm)	A_c	σ_c	Fraction of cells that project pseudopods
	μm^2	$\times 10^3$ dyn/cm^2	
0	16.2	5.3	
	16.2	1.4	
	10.4	2.1	
	34.2	0.61	
		$\bar{\sigma}_c$ = 2.30 ± 2.06	100% (n = 10)
1*	16.2	3.3	
	19.3	3.2	
	12.6	3.4	
	16.2	5.5	
	12.5	4.3	
	18.0	1.2	
	16.2	1.5	
	16.2	1.1	
	7.2	7.4	
		$\bar{\sigma}_c$ = 3.40 ± 2.10	89% (n = 14)
2	13.9	1.5	
	3.5	10.2	
	12.2	3.7	
	7.2	16.1	
	10.8	8.1	
	7.2	12.9	
	10.4	2.2	
	12.2	1.1	
		$\bar{\sigma}_c$ = 6.97 ± 5.71	64% (n = 18)
3–4*	7.2	3.7	
	7.2	6.3	
	12.2	0.90	
	10.3	8.8	
	3.5	7.6	
	3.5	23.0	
	13.2	12.0	
	7.1	0.50	
		$\bar{\sigma}_c$ = 8.90 ± 7.15	50% (n = 10)

*The median values for the 1 and the 3–4 microsphere cases were compared by a Mann-Whitney test, P = 0.567.

actual measurements for a wide range of microsphere diameters, there is little evidence for significant cytoplasmic swelling. This impression is also supported by direct cell dimension measurement under the high resolution light microscope where the cell diameter can be estimated to within ~0.2–0.3 μm. The second assumption (*b*) is probably well-satisfied, since each internalized microsphere remains surrounded by membrane during the course of the experiment and since microspheres did not appear to be released after internalization. (*c*) Since neutrophils are relatively short-lived "end cells" incapable of cell division and contain a nonrenewable population of granules (Hirsch and Cohn, 1960), there is little evidence to suggest that plasma membrane is synthesized during the incubation period. Membrane synthesis and membrane recycling from phagolysosome to plasmalemma have been documented for macrophages after several hours of prescribed incubation conditions (Muller et al., 1980). Macrophages, however, are longer-lived cells compared to neutrophils and have the ability to synthesize new primary lysosomes and lysosomal enzymes (Cohn and Benson, 1965).

The phagocytic capacities measured for a wide range of latex microspheres (Fig. 4) are comparable to those reported in the literature (Table I). The theoretical predictions are remarkably close to the experimental phagocytic capacities provided degranulation across the plasma membrane is accounted for. The morphometric data obtained from electron micrographs clearly support this conclusion, yielding granule to cytoplasmic volume ratios of 8.6% and 10.8% for the 2.0- and 4.0-μm microspheres, respectively. These ratios are substantially lower than those measured for cells incubated with no microspheres, 22.4%. A comparison with granule morphology data from neutrophils that have phagocytosed zymosan (Hoffstein et al., 1974) show similar trends: 22.6% for resting cells and 9.2% for zymosan-filled cells.

By assuming a mean granule surface area of 0.20 μm^2 and a volume of 0.014 μm^3 (Schmid-Schönbein and Chien, 1988), it was estimated that neutrophils filled to phagocytic capacity with 2.0-μm microspheres can release as many as 5,700 granules, thereby adding 1,140 μm^2 of plasma membrane area. A smaller number was estimated for neutrophils filled with 4.0-μm microspheres, 4,800 granules. When this additional membrane area is added to the initial surface area, the prediction for the number of internalized microspheres falls within 13% of the experimentally determined phagocytic capacities for the four larger microsphere diameters. Predictions for the 0.5-μm microsphere, however, overestimate the mean capacity measured by the stereological method. The discrepancy could be due to inhomogeneous distribution of microspheres throughout the cell volume. Thus, the assumption of a random distribution as required by the stereological technique, may not be fully satisfied. Another possible source of error is revealed by the electron micrographs, which shows that phagosomal vacuoles are often filled with several of the smaller 0.5-μm microspheres. In such cases less plasma membrane is used to surround the microspheres than predicted by the single particle–vacuole assumption. Data from the cell aspiration procedure (Fig. 7) show that plasma membrane area is conserved for cells below the phagocytic capacity predicted by Eq. 6.

The observation of an inverse correlation between the quantity of granules released and the number of internalized microspheres (Fig. 6) suggests that degranulation is directly related to the quantity of material engulfed, in agreement with previous observations (Hirsch and Cohn, 1960; Wright and Malawista, 1972). This increased degranulation also correlates with the increased metabolic activity of phagocytically stimulated cells. Electron micrographs of cells in the process of phagocytosis show that

granules are often released into large secondary lysosomes that are positioned proximal to the plasma membrane (Fig. 5 *a*). Granules were also observed in the process of membrane fusion to the plasma membrane (Fig. 5 *b*). Several investigators have observed "regurgitation during feeding" by human neutrophils undergoing phagocytosis of zymosan (Wright and Malawista, 1972) and from stimulation by immune complexes and other particles (Weissmann et al., 1971). Hoffstein et al. (1982) measured a reduction of 195 $\mu m^2 \pm 95$ μm^2 of granule membrane, an amount approximately equal to the expanded cell surface. Bowers et al. (1981) estimated the surface area in phagocytosing amoeba and found that membrane area was replaced on the cell surface during phagocytosis. They concluded that a pool of small vesicles provided exactly the membrane area needed to satisfy cell volume and area conservation.

An interesting aspect of granule release is that during phagocytosis of microspheres, the majority of the granules undergo exocytosis so that their membrane is incorporated into the cell plasma membrane. In contrast, during phagocytosis of bacteria a considerable number of granules incorporate their membrane into the phagolysosome (Hirsch and Cohn, 1960). This suggests that the phagolysosome motion is not a random Brownian phenomenon but is controlled by specific reactions with the cell actin, microfilaments, or with the intracellular membranes.

In a random population of neutrophils, as many as 40% of the cells are not activated and show no phagocytosis. This proportion may be expected from a freshly drawn blood sample (Stavem and Dahl, 1984). Two additional subgroups may be identified from our neutrophil population. The first group constituted as many as 25%, which stopped the engulfment process well below the amount expected based on membrane limitation (Fig. 4). This feature cannot be accounted for by a lack of adhesion, a conclusion in agreement with Bestermen et al. (1982), who showed that surface binding of radiolabeled zymosan to neutrophil plasma membrane remained at its maximal value when internalization ceased. Measurements of the critical pressure required for cell–microsphere separation revealed a greater adhesive stress in cells that were close to their phagocytic capacity. This relationship may be, in part, a matter of contact area formation. Cells that have many surface folds tend not to form a smooth contact area, whereas cells with many microspheres and unfolded membrane may do so more readily. Since phagocytosis is associated with cytoplasmic projection a smooth contact formation may be achieved by active cytoplasmic deformation.

The second subgroup of neutrophils may be identified as those that phagocytose to their membrane capacity. In contrast to the increased adhesion noted for this group, there were distinct limitations in the ability of these cells to form pseudopod projections. Cells in free suspension project fewer and shorter pseudopods with increasing numbers of internalized microspheres (Table II). 50% of the cells in the first subgroup, filled close to their phagocytic capacity, did not exhibit any active cytoplasmic projections in response to mechanical and presumably molecular receptor stimulation. However, 90% of cells with a single microsphere (4.0 μm) exhibit pseudopod projection during physical contact. This may, in part, be due to the fact that in this experiment, surface receptors after internalization are not returned to the plasma membrane, but remain on the membrane surrounding the internalized microspheres, whereas the new membrane provided by the lysosomes does not carry these receptors. This situation would result in a progressive diminuation of surface receptors during phagocytosis. The other possibility that pseudopod formation in the second subgroup of cells is inhibited may be related to the cell's metabolism and energy stores. On the one hand, it should be noted that neutrophils from venous blood suspended in buffer solution without microspheres can form pseudopods for 1–2 d and continuously deform their highly viscous cytoplasm. This suggests that there may be large energy stores in the form of cytoplasmic glycogen. On the other hand, phagocytosis appears to be associated with additional processes than pseudopod formation, i.e., an oxygen burst and endocytosis processes whose energy requirements are not known quantitatively in these cells. The overall process may require more energy than just actin polymerization to form pseudopods and may therefore deplete some cells rapidly.

One of the major factors that determines the size of pseudopods is the available membrane area. Cells that have no excess membrane area do not project pseudopods outwards but instead they grow inward (Schmid-Schönbein and Skalak, 1984). In this case the membrane tension opposes the outward projection of the actin cytoplasmic matrix. We are impressed by the heterogeneity in the pseudopod lengths for the neutrophil populations observed. Although some cells were subject to membrane limitation, other cells were present that continued to project many small discrete pseudopods around the microspheres despite the fact that their membrane area had reached phagocytic capacity and full engulfment was not possible.

The phenomenon of membrane limitation has a number of implications with respect to phagocytosis encountered in other situations. This study was limited to phagocytosis in free suspension. If the cells are instead attached to a substrate, the spreading of the cell by itself will reduce the excess membrane area available for engulfment. Under such conditions smaller amounts of microsphere internalization are expected. Cells that are fully spread out under conditions of strong adhesion may show no phagocytosis at all. Such a situation can be observed regularly for cells that have spread out on the coverslip in the cell observation chamber. Finally, another consequence of membrane limitation may be that phagocytosis of larger microorganisms

in vivo in capillaries is unlikely to occur since in such vessels the cells are elongated, their membrane is unfolded, and little excess plasma membrane area is available.

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