Functional Activity of Enucleated Human Polymorphonuclear Leukocytes

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ABSTRACT Enucleated human polymorphonuclear leukocytes (PMN) were prepared by centrifuging isolated, intact PMN over a discontinuous Ficoll gradient that contained 20 μM cytochalasin B. The enucleated cells (PMN cytoplasts) contained about one-third of the plasma membrane and about one-half of the cytoplasm present in intact PMN. The PMN cytoplasts contained no nucleus and hardly any granules. The volume of the PMN cytoplasts was about one-fourth of that of the original PMN. >90% of the PMN cytoplasts had an "outside-out" topography of the plasma membrane.

Cytoplasts prepared from resting PMN did not generate superoxide radicals (O_2^-) or hydrogen peroxide. PMN cytoplasts incubated with opsonized zymosan particles or phorbol-myristate acetate induced a respiratory burst that was qualitatively (O_2 consumption, O_2^- and H_2O_2 generation) and quantitatively (per unit area of plasma membrane) comparable with that of intact, stimulated PMN. Moreover, at low ratios of bacteria/cells, PMN cytoplasts ingested opsonized *Staphylococcus aureus* bacteria as well as did intact PMN. At higher ratios, the cytoplasts phagocytosed less well. The killing of these bacteria by PMN cytoplasts was slower than by intact cells. The chemotactic activity of PMN cytoplasts was very low.

These results indicate that the PMN apparatus for phagocytosis, generation of bactericidal oxygen compounds, and killing of bacteria, as well as the mechanism for recognizing opsonins and activating PMN functions, are present in the plasma membrane and cytosol of these cells.

Polymorphonuclear leukocytes (PMN[1]) ingest, kill, and degrade invading microorganisms. For this purpose, PMN produce large quantities of hydrogen peroxide and secrete large amounts of granular enzymes into phagocytic vacuoles (1–3). The precise role of these cellular products in the killing process is not known. The importance of the hydrogen peroxide has been deduced from the inability of PMN from patients with chronic granulomatous disease to kill microorganisms that do not themselves secrete hydrogen peroxide (4). Moreover, many types of bacterium are killed only to a minor extent by PMN at low oxygen pressure (5). The importance of the granular enzyme myeloperoxidase has been inferred from the powerful antimicrobial activity of isolated myeloperoxidase with hydrogen peroxide and a halide such as chloride (6), and from the retarded killing of certain microorganisms by myeloperoxidase-deficient PMN (7).

From human PMN, we have prepared cytoplasmic vesicles that contain neither nucleus nor granules. These so-called PMN cytoplasts were obtained from nonstimulated PMN, excluded vital dyes, were osmotically resistant, and did not generate hydrogen peroxide unless incubated with opsonized particles or soluble stimuli. Moreover, these PMN cytoplasts ingested and killed *Staphylococcus aureus*, proving that neither nucleus nor granules are essential for the killing of these bacteria.

MATERIALS AND METHODS

Materials: A 40% (wt/vol) stock solution of Ficoll (Ficoll 70, Pharmacia Fine Chemicals, Uppsala, Sweden) was made in 0.025 M Tris-HCl, pH 7.4. The osmolarity of this stock solution was 296 mosM. Final solutions used for the gradient were made by diluting this stock solution with phosphate-buffered saline (PBS; 140 mM sodium chloride, 10 mM sodium phosphate; pH 7.2).

Cell Isolation: PMN were isolated from the buffy coat of 500 ml of fresh blood from volunteer donors, as described by De Boer et al. (8). The PMN were suspended in a medium that consisted of 138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.6 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.5

[1] *Abbreviations used in this paper:* HMP, hexose monophosphate; PMN, polymorphonuclear leukocytes.

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mM glucose, and 0.5% (wt/vol) human albumin (incubation medium). The final cell suspension contained at least 95% PMN; the remaining cells were lymphocytes.

Enucleation: We used a modification of the method described by Wigler and Weinstein (9) for preparing enucleated PMN. PMN were suspended in 12.5% (wt/vol) Ficoll solution (sp gr d^{25}, 1.0477 g/cm^3; refractive index n_D^{25}, 1.352) with 20 μM cytochalasin B (Sigma Chemical Co., St. Louis, MO). This cell suspension ($\sim 10^8$ PMN/ml) was preincubated for 5 min at 37°C. The suspension (4.5 ml) was then layered on a prewarmed (2 h at 37°C), discontinuous density gradient (4.5 ml of 16% [wt/vol] Ficoll [d^{25}, 1.0578; n_D^{25}, 1.356] on top of 4.5 ml of 25% [wt/vol] Ficoll [d^{25}, 1.0855; n_D^{25}, 1.369). Cytochalasin B (20 μM) was present throughout the gradient. Polycarbonate centrifuge tubes (2.5 × 8.9 cm; Beckman Instruments Inc., Palo Alto, CA) were used. The gradients were centrifuged for 30 min at 81,000 *g* (middle of the tubes) and 33°C in an ultracentrifuge (Kontron Electronic, Inc., Ultracentrifuge TGA 50, Zurich, Switzerland), of which the SW-27 swing-out rotor (Beckman Instruments, Inc.) had been prewarmed for 4 h at 37°C.

After centrifugation, a band of enucleated PMN (band *1*) was present at the interface of the 12.5 and 16% Ficoll solutions. We found a second band at the interface of the 16 and 25% Ficoll solutions; this band *2* contained a few intact PMN and some cell debris. The nuclei were pelleted at the bottom of the tubes (band *3*). The gradient fractions were washed five times with incubation medium to wash away the cytochalasin B (centrifugation: 10 min, 600 *g*, room temperature). For enzyme assays, the gradient fractions and the original PMN were sonicated three times (10 s each, with intervals of 15 s in ice; amplitude 8 μm, frequency ~23 kHz). For metabolic and functional assays, the enucleated PMN were counted electronically (Coulter Counter, Coulter Electronics, Ltd., Dunstable, United Kingdom) in the absence of saponin.

Dimensions: The volume of the enucleated PMN (cytoplasts) was determined with a Coulter Counter (model ZF, Coulter Electronics, Ltd.) equipped with a pulse-height analyzer (Channelyzer, model C-1000, Coulter Electronics Ltd.). Purified erythrocytes (87 femtoliter), lymphocytes (230 femtoliter), and PMN (450 femtoliter) from normal human blood were taken as reference values (10).

We determined the relative surface areas of PMN and PMN cytoplasts with the same equipment after the cells swelled in various hypotonic NaCl solutions (for conditions, see Fig. 2). The volume of the cells increased at decreasing osmotic values of the NaCl solutions. In the solution with the lowest osmotic value that the cells could endure without lysing, the cells were at their largest volume. In this situation, we considered the cells to be spheres. On the basis of these cell volumes the radius and the surface area were calculated.

Marker Assays: We used lactate dehydrogenase (L-lactate: NAD oxidoreductase) as a cytoplasmic marker enzyme, lysozyme (*N*-acetylmuramide glycanohydrolase) as a marker enzyme for all granules, and β-glucuronidase (β-D-glucuronide glucuronohydrolase) as a marker enzyme for the azurophilic granules. These enzymes were measured as described before (11). Assays for myeloperoxidase (donor: H_2O_2 oxidoreductase) (12), that were used as a marker enzyme for the azurophilic granules, and catalase (H_2O_2:H_2O_2 oxidoreductase) (13) have also been described before.

Vitamin B_{12}-binding protein, a marker for the specific granules, was measured with a modification of the method described by Gottlieb et al. (14). Lysate (125 μl) of 15 × 10^4 cells was mixed with 250 μl of vitamin B_{12} solution (2 ng vitamin B_{12} and 0.5 ng ^{57}Co-vitamin B_{12}/ml in 154 mM NaCl solution; Merck Sharp and Dohme, Haarlem, The Netherlands) and incubated for 30 min at 20°C. Then, 500 μl of a suspension of activated charcoal in albumin (8 mg activated charcoal/ml, 0.2% [wt/vol] bovine serum albumin in 154 mM NaCl solution) was added to absorb the free vitamin B_{12}. The suspension was incubated for 10 min at 20°C. The charcoal was spun down at 1,200 *g* for 10 min, a 500-μl sample was taken from the supernate, and the amount of ^{57}Co-vitamin B_{12} in it was measured in a gamma-radiation counter. The values measured were compared with a sample that had not been treated with charcoal, and were expressed as percentage binding.

Alkaline phosphatase (orthophosphoric monoester phosphorylase) was taken as a marker of the plasma membrane. This enzyme was measured with a modification of a method described by Bessey et al. (15). A 100-μl sample, which contained material from ~10^6 PMN, was added to 0.9 ml of buffered *p*-nitrophenylphosphate solution (5 mM sodium acetate, 5 mM $MgCl_2$, 5 mM glycine-potassium hydroxide, pH 9.0, 65 mM KCl, and 5 mM *p*-nitrophenylphosphate). After 1 h, the incubation at 37°C was stopped with 3 ml of ice-cold 0.1 M NaOH. The tubes were centrifuged and the absorbance of the liberated *p*-nitrophenol was measured in the supernate at 405 nm. Values obtained with substrate only were subtracted from the values obtained with cell-derived material.

DNA was measured with the method described by Labarca and Paigen (16). A 20-μl sample (containing material from ~8 × 10^4 PMN) was added to 1,950 μl of Hoechst 33258 solution (Calbiochem-Behring Corp., La Jolla, CA; 0.1 μg/ml in 2 M NaCl, 50 mM $NaHPO_4$, and 2 mM EDTA, pH 7.4). The fluorescent complex between DNA and Hoechst 33258 was measured in a fluorimeter (excitation, 356 nm; emission, 458 nm). The DNA concentration in the samples was calculated by comparison with a set of calf thymus DNA standards of known concentrations (Worthington Biochemical Corp., Freehold, NJ).

We measured protein according to the method of Bradford (17), because the presence of Ficoll interferes with the method of Lowry. A 20-μl sample, containing maximally 500 ng of protein/ml, was added to 200 μl of PBS and 2 ml of Coomassie Brilliant Blue G250 (100 mg in 1 liter 5% [vol/vol] ethanol, 8.5% [wt/vol] H_3PO_4). The absorbance of the protein/Coomassie Brilliant Blue complex was measured at 595 nm. The amount of protein per sample was calculated by comparison with a standard curve of known amounts of bovine albumin.

Reaction with Antibodies: PMN (10^6) or PMN cytoplasts (3 × 10^6) were fixed with 0.5% (wt/vol) paraformaldehyde for 5 min at room temperature and washed twice with PBS. The cells were then incubated for 30 min at room temperature with 1:1,000 dilutions of the monoclonal mouse IgG1 antibodies B13-3 (30 mg of protein/ml; directed against a PMN plasma membrane antigen with a molecular weight of 87,000) or C17 (20 mg of protein/ml; directed against the thrombocyte plasma membrane antigen glycoprotein IIIa). Both antibodies were kindly provided by Dr. P. Lansdorp from our institute. After two washings with PBS, the cells were stained with a 1:80 dilution of a 11.8-mg protein/ml fluorescein isothiocyanate (FITC)-labeled goat-anti-mouse IgG (30 min, room temperature), washed twice with PBS, and suspended in 70% (vol/vol) glycerol in PBS. 200 cells were scored as positive or negative by fluorescence microscopy.

Alternatively, the PMN were incubated for 30 min at room temperature with a 1:5 dilution of F(ab') fragments of a rabbit-IgG antiserum against C3b receptor from human erythrocytes (0.6 mg protein/ml; kindly provided by Dr. M. R. Daha, University of Leiden, The Netherlands). F(ab') fragments of rabbit IgG against human IgG were used as a control. The PMN were washed twice with PBS, and part of the cells was then suspended in 12.5% (wt/vol) Ficoll and subjected to the cytoplast preparation (as described previously in this paper). Next, the PMN and the PMN cytoplasts were fixed with 1% (wt/vol) paraformaldehyde and washed twice with PBS. Both preparations were then incubated for 30 min at room temperature with a 1:100 dilution of FITC-labeled F(ab') fragments of swine IgG against rabbit IgG (6 mg protein/ml; Dakopatts, Copenhagen, Denmark), washed twice with PBS, suspended in 70% (vol/vol) glycerol in PBS, and examined by fluorescence microscopy.

For quantitative measurement of B13-3 binding, this antibody was labeled with ^{125}I by the iodogen method (18). In brief, 100 μg of iodogen in 100 μl of dimethyl chloride was put into a 10-ml bottle, and the solvent was evaporated with dry nitrogen gas. Next, 100 μg of B13-3 in 200 μl of PBS plus 1 mCi $Na^{125}I$ was added and incubated for 1 min at room temperature. The supernate was then dialyzed three times against 500 ml of PBS plus 0.01% (wt/vol) potassium iodide. 10^7 PMN or 3 × 10^7 PMN cytoplasts were incubated for 45 min at room temperature with 0.8 ng of the ^{125}I-labeled B13-3 together with 1.5 μg of unlabeled B13-3, in a volume made 300 μl with incubation medium. Thereafter, the PMN and the PMN cytoplasts were washed five times with 10 ml of PBS plus 0.5% (wt/vol) human albumin. The radioactivity was counted in a gamma-radiation counter and expressed as percentage of the radioactivity bound to intact PMN.

Functional Tests: Chemotaxis toward casein was measured with the leading front method of Zigmond and Hirsch (19), essentially as described before (12). Instead of Millipore filters, we used cellulose-nitrate filters (Sartorius, type SM 11302, Göttingen, Federal Republic of Germany). The pore size was 3 μm. Because PMN cytoplasts lack a nucleus, staining was performed with amido black (0.2% [wt/vol], in ethanol 96% [4 parts] plus glacial acetic acid [1 part]). Chemotaxis was also measured using the agarose method of Nelson et al. (20). PMN (3.5 × 10^5) and cytoplasts (3.5 × 10^5 or 1.4 × 10^6) were tested for 18 h against zymosan-activated human serum and against minimal essential medium (MEM).

Phagocytosis and intracellular killing of *S. aureus* (strain "Oxford" no. 421) were measured essentially as described before (12). In the killing assay, extracellular bacteria were not treated with antibiotics, because these agents were found to penetrate the PMN cytoplasts. Instead, the extracellular bacteria were removed by centrifugation (4°C, 10 min, 280 *g*) and washed three times with ice-cold PBS/human-albumin (0.5%, [wt/vol]).

Metabolic Reactions: Oxygen consumption was measured with an oxygen electrode as described by Weening et al. (21). Production of $^{14}CO_2$ from [1-^{14}C]glucose was determined as a measure of the hexose monophosphate (HMP)-shunt activity. A modification of a previously described method (12) was used.

Hydrogen peroxide-production was measured in a direct fluorimetric assay. PMN or PMN cytoplasts were suspended in incubation medium in a fluorescence cuvette to 0.3 × 10^6 or 0.9 × 10^6/ml, respectively, in the presence of 1 mM homovanillic acid (Merck AG, Darmstadt, Federal Republic of Germany),

1.44 U of horse-radish peroxidase (Boehringer, Mannheim, Federal Republic of Germany), 2 mM NaN_3, and 4 μM superoxide dismutase (Sigma Chemical Co.). The cells were stimulated at 37°C with opsonized zymosan particles (0.1 mg/ml) or the soluble stimulator phorbol-myristate acetate (100 ng/ml). The H_2O_2 generated by the cells oxidized the homovanillic acid in a peroxidase-catalyzed reaction to a fluorescent product. The resulting increase in fluorescence was measured (excitation, 315 nm; emission, 420 nm) and calibrated with a set of samples with known amounts of hydrogen peroxide.

Superoxide-anion generation was measured by ferricytochrome-*c* reduction (150 μM horse-heart ferricytochrome *c*, grade III, Sigma Chemical Co.) as previously described (22). The values found in the presence of 1.3 μM superoxide dismutase were subtracted from all other values.

RESULTS

Preparation of PMN Cytoplasts

After centrifugation of PMN over the Ficoll/cytochalasin-B-gradient (see Materials and Methods), three fractions were collected. The top fraction contained the 12.5% Ficoll solution and band *1*, the second fraction contained the 16% Ficoll solution and band *2*, and the bottom fraction contained the 25% Ficoll solution and band *3*. Each fraction (~4.5 ml) was diluted with an equal volume of PBS and centrifuged (700 *g*, 10 min, room temperature). The supernates and the resuspended pellets (in 5 ml of PBS) were separately tested for protein and subcellular markers.

Table I shows that band *1* contained about one-third of all protein and alkaline phosphatase (plasma membrane marker), as well as about one-half of all lactate dehydrogenase (cytoplasmic marker) and ~40% of all catalase activity (not shown). In contrast, this fraction contained no DNA (Table I) or myeloperoxidase (not shown) and hardly any other granular proteins (Table I). Thus, band *1* consists of enucleated PMN (PMN cytoplasts); this was confirmed by electron microscopy (Fig. 1). Similarly, the enzymatic (Table I) and microscopic analyses revealed that band *2* contained some intact PMN and cell debris, and band *3* plasma-membrane vesicles filled with nuclei and granules (PMN karyoplasts).

The diameter of the PMN cytoplasts was 4–8 μm ($n = 25$), measured by electron microscopy, and 6.4 ± 1.2 μm (mean ± SD, $n = 100$), measured by light microscopy. Intact PMN had diameters of 10–15 μm (EM, $n = 25$) and 11.7 ± 2.3 μm (mean ± SD, $n = 100$; light microscopy), respectively.

The volume of the PMN cytoplasts was determined electronically (see Materials and Methods). This value varied between 90 and 120 femtoliter (mean 104 femtoliter, SD 14 femtoliter, $n = 5$). The relative surface area of PMN and cytoplasts was determined after swelling in hypotonic NaCl solutions (see Materials and Methods). PMN cytoplasts increased 40% in volume before lysing; PMN increased ≈100% in volume before lysing. From the largest volumes measured, we calculated a surface ratio between cytoplasts and PMN of 0.29 (mean of two experiments).

The number of cytoplasts recovered from band *1* was always about the same as the number of PMN applied to the gradient. Apparently, each PMN formed one cytoplast. Up to 600 × 10^6 PMN were applied to one gradient (surface area 4.1 cm^2) without signs of overloading (recovery of >1% intact PMN from band *1* or *2*). Centrifugation at forces <81,000 *g* resulted in appearance of intact PMN in band *2*. Centrifugation for 10 or 20 min gave results similar to those obtained with 30-min centrifugation time. Centrifugation at 20°C resulted in formation of functionally intact PMN cytoplasts at ~60% yield compared with the procedure at 33°C. At 4°C, no PMN cytoplasts were formed. Centrifugation of PMN over a Ficoll gradient without cytochalasin B resulted in very low yields of cytoplasts.

Topography

The PMN cytoplasts had an intact plasma membrane, because the vital dye trypan blue was excluded by 99% of the neutroplasts. A similar percentage of the PMN cytoplasts released fluorescein from the vital dye fluorescein diacetate. PMN cytoplasts were less osmotically resistant than intact PMN. Fig. 2 shows that PMN cytoplasts lost 50% of their lactate dehydrogenase when these cells were incubated with ~130 mosM NaCl. Intact PMN had to be incubated with 70 mosM NaCl for a similar reaction.

The plasma membrane of the PMN cytoplasts faced with the original outside outwards. This conclusion was deduced from the observation that similar percentages of cytoplasts and the original PMN bound the monoclonal mouse-IgG1-antibody B13-3 against human PMN and F(ab') fragments of rabbit IgG against C3b receptors of human erythrocytes. The values are given in Table II. Moreover, upon lysis, PMN cytoplasts displayed a similar percentual increase in activity of alkaline phosphatase as did the original PMN (not shown).

Metabolic Activity

Without additions, PMN cytoplasts consumed very little oxygen, did not reduce exogenous cytochrome *c*, and did not release hydrogen peroxide (Table III). When PMN cytoplasts were incubated with opsonized zymosan particles (1 mg/ml) or the soluble stimulator phorbol-myristate acetate (100 ng/ml), a sharp increase in the consumption of oxygen was observed (Table III). This increased respiration was accompanied by a concomitant reduction of exogenous cytochrome *c* and release of hydrogen peroxide. When the activity was expressed per unit of alkaline phosphatase, as a measure of the amount of plasma membrane, the PMN cytoplasts displayed a metabolic activity comparable with that of intact PMN (Table III). The time course of these reactions was similar for PMN and cytoplasts (Figs. 3 and 4). PMN that had been incubated with Ficoll plus cytochalasin B and washed in the same way as the cytoplasts were as active as untreated PMN (not shown).

The activity of the HMP shunt was also measured. Intact PMN, incubated either with opsonized zymosan particles or with phorbol-myristate acetate, produced ~15 times more $^{14}CO_2$ from [1-^{14}C]glucose than did resting cells (Table IV). PMN cytoplasts did likewise, and displayed activity similar to intact PMN, when expressed per unit of either lactate dehydrogenase or glucose-6-phosphate dehydrogenase (as a measure of the amount of cytoplasm) (Table IV).

Functional Activity

Chemotaxis was measured by the distance of cell penetration into a filter with pores of 3 μm, and by the distance of cell migration under agarose (see Materials and Methods). Table V shows that treatment of PMN with Ficoll (12.5% [wt/vol]) plus cytochalasin B (20 μM) at 33°C, for the same period as used for the cytoplast preparation, followed by washing five times with incubation medium, inhibited the chemotactic capacity of the cells 10–35%. PMN cytoplasts showed ~20% of the activity of cytochalasin B-treated cells toward casein but no activity at all toward zymosan-activated serum.

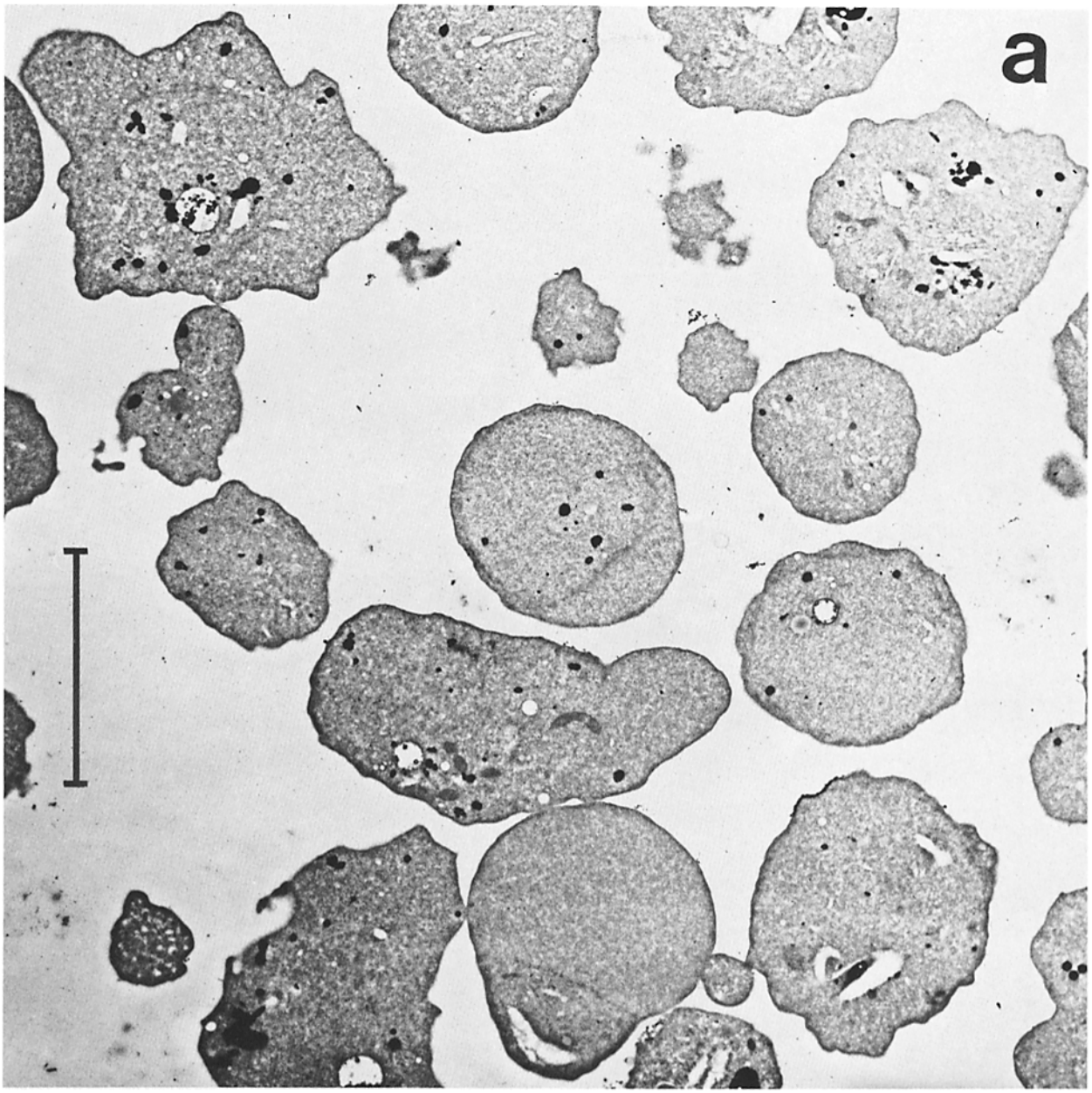

FIGURE 1 Electron micrograph of PMN cytoplasts and PMN. Cytoplasts (*a*) and the original PMN (*b*) were fixed, stained for peroxidase activity, postfixed, embedded, cut, and stained with uranyl acetate and lead citrate, as described by Beelen et al. (45). Note the lack of peroxidase activity in the PMN cytoplasts. Bar, 5 μm. × 7,000 (*a*); × 5,000 (*b*).

Fig. 5 shows that the PMN cytoplasts ingested *S. aureus* bacteria at about the same rate as did the original PMN at a low ratio of bacteria to cells, but significantly slower at higher ratios. A dose-response curve is shown in Fig. 6. The method employed for this study measures only intracellular bacteria because adhering bacteria are removed by digestion with lysostaphin. Table VI shows that about twice as many cytoplasts as PMN did not ingest any bacteria. Fig. 7 shows that *Escherichia coli* bacteria were also ingested by the PMN cytoplasts.

The intracellular killing of *S. aureus* by the PMN cytoplasts was less efficient than that shown by intact PMN (Fig. 8). Treatment with Ficoll plus cytochalasin B, followed by washing five times, slightly decreased the killing capacity of the neutrophils. Nevertheless, the cytoplasts killed ~40% of the ingested bacteria within 30 min of reincubation at 37°C.

DISCUSSION

Enucleation of PMN by centrifugation over a Ficoll gradient, as described above, is a process that probably involves accumulation of the intact cells at the interface between 16 and 25% Ficoll (band *2*), movement of nuclei and granules to the centrifugal side of the cells, fusion of the plasma membrane around the cytoplasmic (centripetal) and around the karyoplastic (centrifugal) parts of the cells, flotation of the cytoplasts to the interface between 12.5 and 16% Ficoll (band *1*), and sedimentation of the karyoplasts to the bottom of the tubes (band *3*)[2]. This order of events is deduced from the

[2] When intact PMN karyoplasts are needed for functional studies, care should be taken to avoid clumping of these particles, for instance, by putting a very dense layer of Ficoll as a cushion at the bottom of the centrifuge tubes.

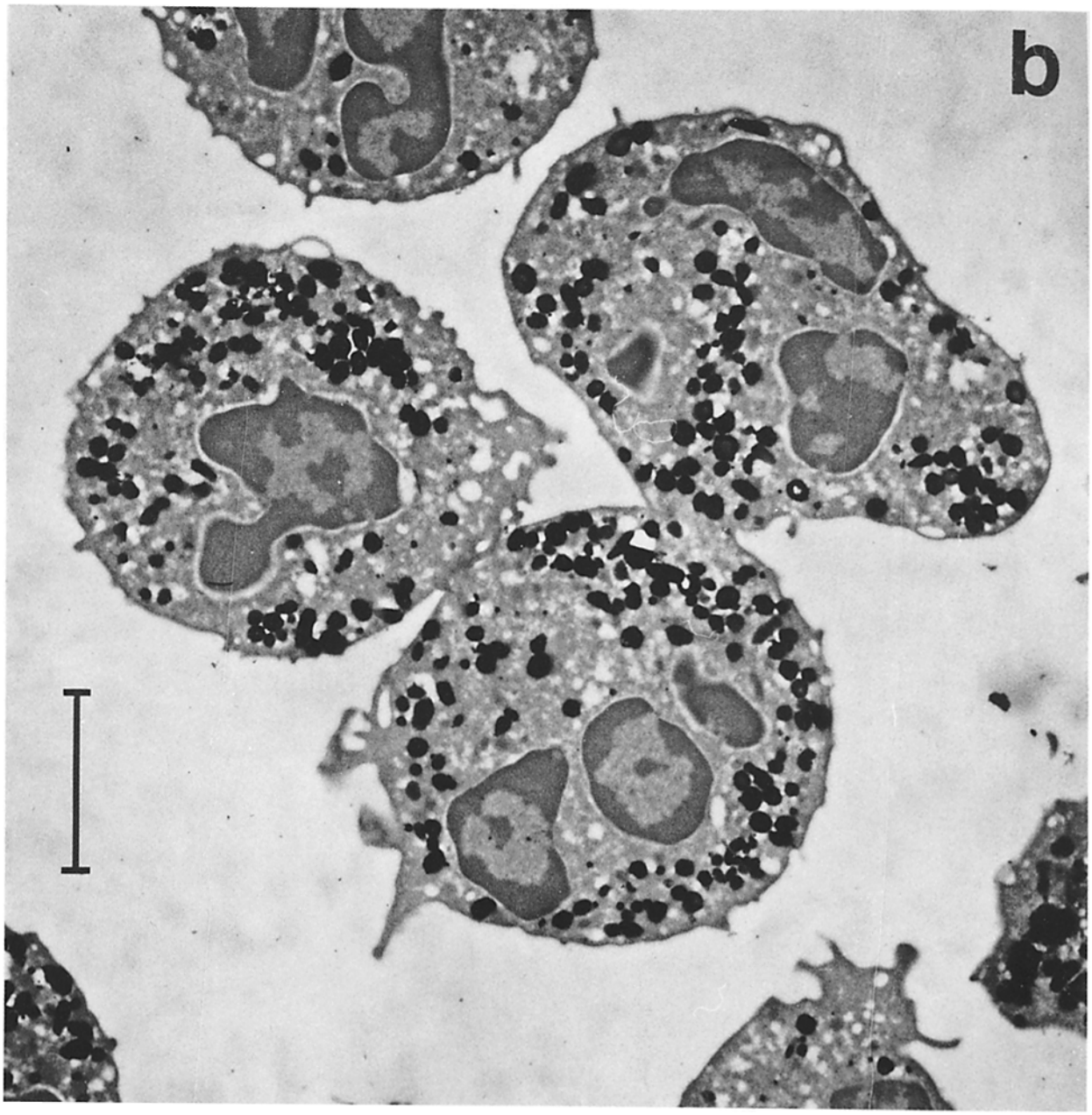

FIGURE 1 *continued*

observations that centrifugation at a force of <81,000 *g* resulted in localization of intact cells in band *2*, whereas centrifugation at 81,000 *g* resulted in practically complete division of the cells into separated cytoplasts and karyoplasts. The conclusion that a PMN cytoplast is "pinched off" from the rest of the cell is based on the low activity of lactate dehydrogenase in the gradient material after the cytoplasts have been formed (Table I), on the structural integrity of the cytoplasts and on the "outside-out" configuration of the cytoplasts (Table II). The presence of cytochalasin B is essential for PMN cytoplast formation. Apparently, the microfilamental structure must be (temporarily) destroyed before the formation and/or separation of cytoplasts and karyoplasts can take place. Similarly, optimal PMN cytoplast formation takes place only at temperatures above the transition temperature of the lipids in the PMN plasma membrane (i.e., 27°C [23]).

According to Table I, the PMN cytoplasts contain 33% of the original amount of alkaline phosphatase in the PMN. Because each PMN forms one cytoplast, this indicates that each cytoplast contains a mean of 33% of the amount of alkaline phosphatase present in its "parent" PMN. Alkaline phosphatase is regarded as a plasma membrane marker of human PMN (24); however, this has been disputed (25). Therefore, we have checked our results with binding of a monoclonal antibody against a PMN membrane antigen. Similar results were obtained with either marker (Table I).

These methods presume, however, that the plasma membrane markers of the PMN are distributed over the cytoplasts and the karyoplasts according to the amount of plasma membrane around these particles, i.e., that the markers are not concentrated on the surface of either particle. Therefore, we also measured the ratio of the surface areas of cytoplasts and intact PMN with an independent method. This was done by electronically measuring the volume of PMN and cytoplasts after maximal swelling in hypotonic media. Assuming that the cells were spherical in media with an osmotic value close

to that causing lysis, we calculated that the mean surface area of PMN cytoplasts was 29% of the mean surface area of PMN.

Thus, all three methods indicate that the cytoplasts contain about one-third of the amount of plasma membrane of the original PMN. This proves two things: (*a*) The plasma membrane markers investigated did not redistribute during the cytoplast preparation; and (*b*) Alkaline phosphatase can be used as a marker to calculate the cytoplasts' activities per unit of surface area. Moreover, from the observation that PMN swelled to almost twice their original volume whereas PMN cytoplast increased only ~40% in volume, we conclude that, in isoosmotic media, the cytoplast surface contains much less folds than that of the PMN. This may be caused by the division of the contents of each PMN over two new particles with the same total amount of plasma membrane.

The volume of the cytoplasts (104 femtoliter) is 23% of the PMN volume. The cytoplasts contain 48% of the original amount of lactate dehydrogenase in the PMN (Table I). Lactate dehydrogenase is a soluble enzyme (26) and is therefore generally regarded as a good marker for the cytoplasm of a cell. Thus, 23% of the volume represents 48% of the cytoplasm of PMN. In other words, the cytoplasm of PMN takes about half of the total volume of these cells; the rest of the volume is occupied by the nucleus and the granules.

The observation that the PMN cytoplasts contain ~40% of the catalase activity of intact PMN is in accord with a previous observation (13) that ~90% of the catalase activity in human PMN is in a soluble form. Indeed, in these cells, catalase appears to be localized both as a soluble enzyme in the cytoplasm (26) and enclosed in small granules (27).

Our finding that cytoplasts prepared from resting PMN do not reduce oxygen to hydrogen peroxide unless stimulated by either opsonized particles or soluble stimuli, deserves special attention. As far as we know, this is the first description of a subcellular preparation of PMN with a fully intact oxidase

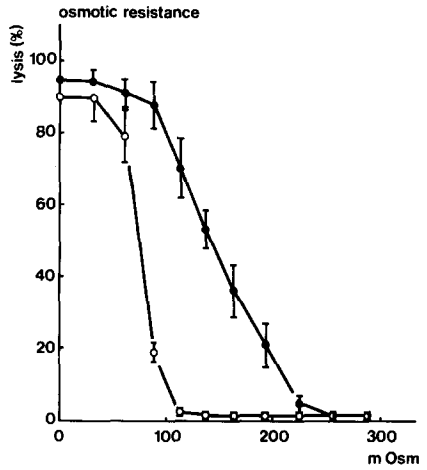

FIGURE 2 Osmotic resistance of PMN and PMN cytoplasts. Cytoplasts and the original PMN were incubated with NaCl solutions of various concentrations for 30 min at 0°C. Thereafter, the incubations were made 140 mM with respect to NaCl, the cells were spun down, and the activity of lactate dehydrogenase was measured in the supernates. The activity of this enzyme in the PMN or the cytoplasts was taken as 100%, and the results were expressed as percentage lysis (mean ± SEM of three paired experiments). O, PMN; ●, PMN cytoplasts.

TABLE I

Biochemical Analysis of Ficoll-gradient Fractions

Marker	Band *1* (top)	Band *2* (middle)	Band *3* (bottom)	Total recovery
Protein	31 ± 2	13 ± 2	48 ± 5	102 ± 1
DNA	0.5 ± 0.3	10 ± 3	93 ± 0.1	104 ± 3
Alkaline phosphatase*	33 ± 2	15 ± 3	35 ± 3	95 ± 2
Lactate dehydrogenase	48 ± 2	11 ± 2	27 ± 1	95 ± 2
Lysozyme	2 ± 0.2	5 ± 2	80 ± 5	96 ± 3
β-glucuronidase	6 ± 2	8 ± 2	77 ± 6	96 ± 1
Vitamin-B_{12}-binding protein	2 ± 0.2	5 ± 1	79 ± 2	103 ± 4

Results in percentage of values found with intact PMN (mean ± SEM of three paired experiments). Total recovery was calculated as the sum of all values found in the supernates and the pellets (bands) of each fraction. In the original PMN, the following values were found (mean ± SD per 10^6 PMN, $n = 3$): protein, 43 ± 6 μg; DNA, 4.9 ± 1.3 μg; alkaline phosphatase, 0.74 ± 0.18 mU; lactate dehydrogenase, 38 ± 6 mU; lysozyme, 3.7 ± 1.1 μg; β-glucuronidase, 1.2 ± 0.3 mU; and vitamin B_{12}-binding protein, 1.1 ± 0.6 ng vitamin B_{12} bound.

* Results obtained with alkaline phosphatase as a plasma membrane marker were checked with a binding assay of ^{125}I-labeled monoclonal antibody B13-3 (see Materials and Methods). PMN cytoplasts (band *1*) bound 36% ± 5% (mean ± SD, $n = 5$) of the amount of antibody bound by intact PMN.

TABLE II

Reaction of PMN Cytoplasts with Antibodies Against PMN

Cell preparation	B13-3	C17	F(ab') anti-C3b-receptor	F(ab') anti-IgG
PMN	99	9	93	0
PMN + cyto B	100	7	92	1
PMN cytoplasts	96	9	90	0

* PMN and PMN cytoplasts were incubated with the monoclonal mouse IgG antibodies B13-3 (against human neutrophils) or C17 (against glycoprotein IIIa of human thrombocytes) and stained with FITC-labeled goat-anti-mouse IgG, as indicated in Materials and Methods. Alternatively, PMN were incubated with F(ab') fragments of either rabbit IgG against C3b receptors of human erythrocytes or rabbit IgG against human IgG; from part of these PMN, cytoplasts were prepared, and both preparations were stained with FITC-labeled F(ab') fragments of swine IgG against rabbit IgG (see Materials and Methods). Data are given as percentage of cells scored positive with fluorescence microscopy (200 cells counted; mean of two paired experiments). PMN + cyto B: PMN incubated with 12.5% (wt/vol) Ficoll plus 20 μM cytochalasin B for 30 min at 33°C and washed five times with PBS (as were PMN cytoplasts).

TABLE III

Oxidative Metabolism of PMN and PMN Cytoplasts

Parameter	PMN			PMN cytoplasts		
	At rest	+STZ	+PMA	At rest	+STZ	+PMA
Oxygen consumption	0.7 ± 0.1	16.0 ± 1.8	11.0 ± 1.5	0.6 ± 0.2	9.0 ± 1.3	9.1 ± 1.9
Hydrogen peroxide production	0	6.1 ± 1.0	6.4 ± 1.0	0	4.1 ± 0.8	6.2 ± 1.3
Cytochrome *c* reduction	0	6.0 ± 0.4	7.3 ± 1.9	0	8.0 ± 1.2	9.3 ± 1.5

Values (rates at linear part of curves, see Figs. 3 and 4) in μmol per unit alkaline phosphatase per min (mean ± SEM, 4–11 paired experiments). STZ, serum-treated zymosan particles; and PMA, phorbol-myristate acetate. PMN incubated with 12.5% (wt/vol) Ficoll plus 20 μM cytochalasin B for 30 min at 33°C and washed five times with PBS (as were PMN cytoplasts) displayed reactions similar to those of untreated PMN (not shown).

system. So-called podosomes have to be prepared from stimulated PMN to show NADPH-oxidase activity; podosomes made from resting PMN cannot be activated (28). Probably, the activation mechanism of the oxidase is destroyed in these particles. Phagocytic vesicles, by definition, are always prepared from stimulated PMN (29).

Our results indicate that neither nucleus nor granules are needed for the oxidase activity or for "triggering" of this reaction. Upon hypotonic lysis, ultrasonic disruption, or detergent treatment of the PMN cytoplasts, either before or after stimulation, we found that very little production of hydrogen peroxidase was left, even in the presence of 1 mM NADPH (not shown). It is highly probable, therefore, that the oxidase system is localized in the plasma membrane and uses cytoplasmic substrate(s) for its activity. This conclusion agrees with observations by other investigators (24, 26, 28–30) but contradicts reports on a possible granular component of the oxidase system (31–33). Probably, upon disruption of the plasma membrane, the molecular configuration of the oxidase system is destroyed or a soluble cytoplasmic factor is lost.

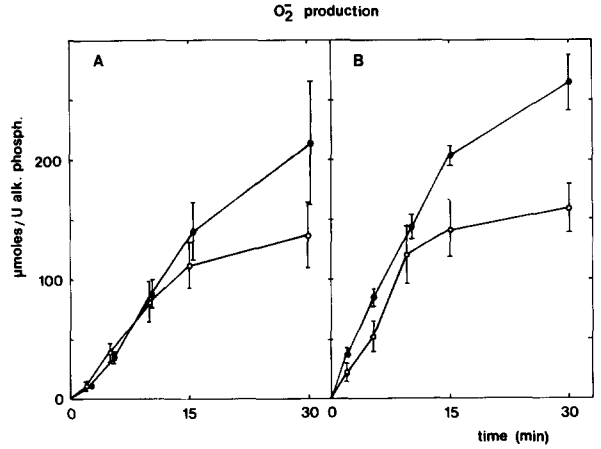

FIGURE 3 Superoxide production by PMN and PMN cytoplasts. Cytoplasts and the original PMN were incubated with serum-treated zymosan (*A*) or phorbol-myristate acetate (*B*). The reduction of cytochrome c (see Materials and Methods) was measured in cell-free supernates at 550 nm. Values in micromoles of cytochrome c reduced per unit of alkaline phosphatase (mean ± SEM of seven (*A*) or four (*B*) paired experiments). O, PMN; ●, PMN cytoplasts.

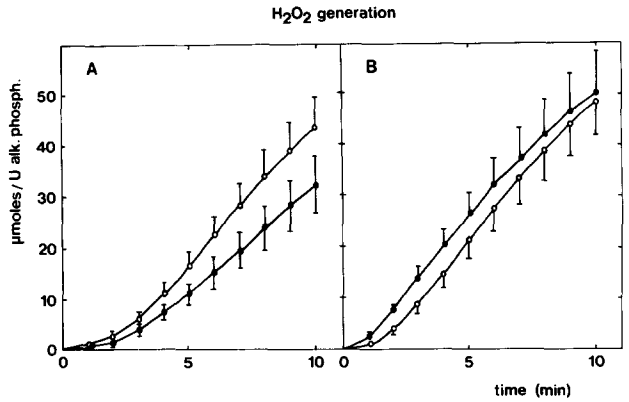

FIGURE 4 Hydrogen peroxide generation by PMN and PMN cytoplasts. Cytoplasts and the original PMN were incubated with serum-treated zymosan (*A*) or phorbol-myristate acetate (*B*). The oxidation of homovanillic acid (see Materials and Methods) was measured fluorimetrically. Values in micromoles of H_2O_2 generated per unit of alkaline phosphatase (mean ± SEM of 11 (*A*) or 8 (*B*) paired experiments). O, PMN; ●, PMN cytoplasts.

Table III shows that PMN cytoplasts stimulated by opsonized zymosan produced ~70% of the hydrogen peroxide generated by intact PMN (per unit area of plasma membrane). In contrast, phorbol-myristate acetate induced the same H_2O_2 generation in PMN cytoplasts and in PMN. Thus, the pathway of oxidase stimulation by IgG and/or particulate activators of the alternative complement pathway (34) might have been slightly damaged in the PMN cytoplasts, whereas the pathway of oxidase stimulation by phorbol-myristate acetate (35) is still fully intact. For this reason, and also because of the low activity of granular enzymes, PMN cytoplasts should constitute very good starting material for preparing functional plasma membranes from PMN.

Table III also shows that PMN cytoplasts had conserved the ability to reduce extracellular cytochrome *c* in a superoxide-dismutase-inhibitable reaction even better than the ability to generate hydrogen peroxide. In fact, the cytochrome *c*-reduction of the cytoplasts was greater (per unit area of membrane) than that of the original PMN, although the oxygen consumption was lower. In our opinion, this means that the oxidase in PMN cytoplasts is slightly distorted, which enables cytochrome *c* (and superoxide dismutase) to react with a superoxide adduct of the oxidase enzyme more efficiently than in intact PMN. A similar but much larger effect has previously been found with PMN homogenates (36). Therefore, we regard the ability of an oxidase preparation to generate hydrogen peroxide as a better indication of its activity than its ability to reduce cytochrome *c*.

Although the PMN cytoplasts contain hardly any granules, their oxidase activity terminated 15–30 min after its activa-

TABLE V

Chemotaxis of PMN and PMN Cytoplasts

	Casein		Zymosan-activated serum	
	−	+	−	+
PMN	36 ± 4	105 ± 5	777 ± 17	2,230 ± 57
PMN + cyto B	29 ± 3	94 ± 6	563 ± 23	1,497 ± 105
PMN cytoplasts	15 ± 2	29 ± 2	107 ± 15	117 ± 12

Distances traveled by cells in microns (mean of nine paired experiments ± SEM). PMN + cyto B, see legend to Table II.

TABLE IV

HMP Shunt Activity of PMN and PMN Cytoplasts

	PMN			PMN cytoplasts		
	At rest	+STZ	+PMA	At rest	+STZ	+PMA
(1)	0.02 ± 0	0.33 ± 0.05	0.53 ± 0.03	0.03 ± 0.01	0.29 ± 0.06	0.35 ± 0.08
(2)	0.01 ± 0	0.17 ± 0.03	0.27 ± 0.01	0.02 ± 0.01	0.15 ± 0.03	0.18 ± 0.04

Values in μmol of glucose metabolized through the HMP shunt per 30 min (1) per unit of glucose-6-phosphate dehydrogenase (G6PD) or (2) per unit of lactate dehydrogenase (LDH) (mean ± SEM, five paired experiments). PMN contained 21.6 ± 6.3 mU of G6PD and 42.4 ± 10.6 mU of LDH per 10^6 cells; cytoplasts contained 8.6 ± 3.1 mU of G6PD and 16.9 ± 5.2 mU of LDH per 10^6 cells (mean ± SD, n = 5). For further information, see legend to Table III.

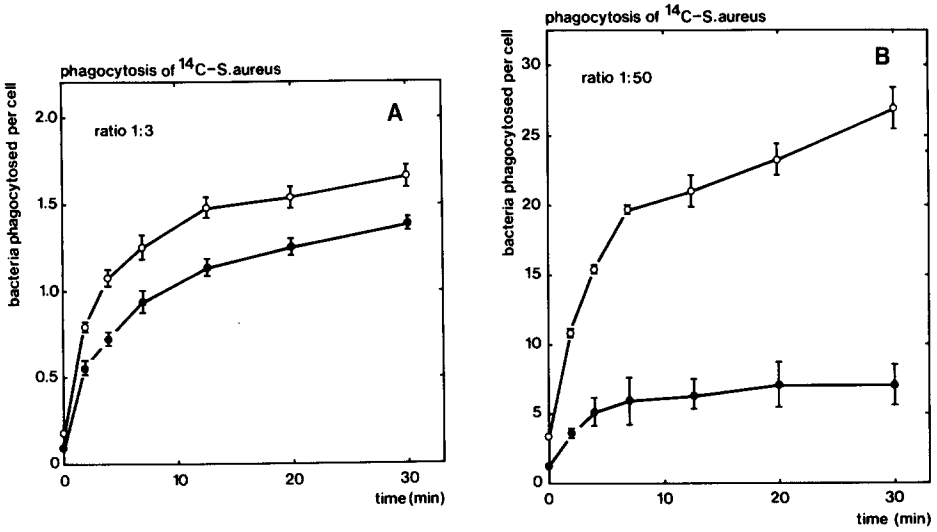

FIGURE 5 Phagocytosis of *S. aureus* vs. time. Cytoplasts or the original PMN were mixed with three times as many (*A*) or 50 times as many (*B*) ^{14}C-labeled *S. aureus* bacteria in 10% (vol/vol) human AB serum. At various times, samples were taken, treated with lysostaphin, centrifuged, washed, and counted for radioactivity. The results are expressed as number of bacteria phagocytosed per PMN (O) or per cytoplast (●) (mean ± SEM of three paired experiments).

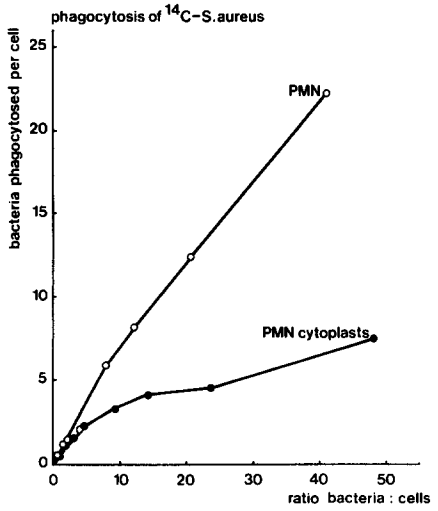

FIGURE 6 Phatocytosis of *S. aureus* vs. bacteria/cell ratio. The cells were incubated as described in the legend of Fig. 5, for 30 min. Results in number of bacteria phagocytosed per PMN (O) or per cytoplast (●).

TABLE VI
Microscopic Evaluation of S. aureus Phagocytosis

	No. of bacteria per cell				
	0	1–3	4–5	6–10	>10
PMN	11 ± 1	12 ± 2	14 ± 1	19 ± 1	45 ± 1
PMN cytoplasts	18 ± 3	28 ± 3	14 ± 1	17 ± 2	23 ± 1

Values in percentage of cells examined (mean ± SEM of four paired experiments). PMN and PMN cytoplasts were incubated for 30 min at 37°C with 15 opsonized *S. aureus* bacteria per cell, treated with lysostaphin, and washed (12). Thereafter, the number of bacteria was counted microscopically in 100 cells.

tion, as is found in intact neutrophils (37). Thus, the termination of the oxidase activity is not (only) due to inactivation of the oxidase system by myeloperoxidase or other granular enzymes (38). Possibly, (per)oxidation of the oxidase system or its phospholipid environment by H_2O_2 itself contributes to this inactivation (39).

At low ratios of bacteria/cells (<5), the PMN cytoplasts ingested as many *S. aureus* and as rapidly as did intact PMN (Fig. 5*A*). However, at higher ratios, the cytoplasts were slower and stopped when they had ingested a mean of seven bacteria per cell (Figs. 5*B* and 6). Microscopic examination (Table VI) revealed that ~80% of the cytoplasts took part in this reaction (for PMN, this value was ~90%). These differences may have been caused by the preparation procedure, the smaller size, and/or the lower content of microfilaments in the cytoplasts.

PMN cytoplasts displayed hardly any chemotactic activity. It might be that in PMN cytoplasts the microfilamental system or other elements critical for cell movement are present at concentrations too low to enable these cells to move at a rate comparable with that of intact PMN. The normal chemotactic response of cytokineplasts, disconnected from heat-treated PMN, to an erythrocyte destroyed by laser irradiation (40, 41) may be explained either by the strong chemotactic signal emitted by the target or by the fact that the cytokineplasts contain the bulk of the PMN's contractile protein (41).

The killing of *S. aureus* by PMN cytoplasts is quite surprising. Although it is known that PMN lacking myeloperoxidase are able to kill these bacteria in vitro (42), it is generally assumed that bactericidal oxygen products cooperate with granular components in the killing of bacteria. Thus, it has been postulated that myeloperoxidase (6, 7), lysozyme (43), and/or lactoferrin (44) assist in bactericidal reactions. Our experiments prove that these assumptions are not necessarily correct, because PMN cytoplasts contain hardly any granules but nevertheless killed *S. aureus* to an appreciable extent. Possibly, the concentration of hydrogen peroxide (or perhaps other oxygen metabolites) in the phagosomes reaches values that are bactericidal already in themselves. We are well aware, however, that this need not be true for bacteria other than *S. aureus*. In fact, we have indications that the perforation and killing of *E. coli* is severely decreased in PMN cytoplasts as compared with intact PMN. This is at present under investigation. Moreover, we expect that the degradation of bacterial proteins will be strongly depressed in PMN cytoplasts. This, too, might limit the bactericidal efficacy of the cytoplasts.

In conclusion, we have found that PMN vesicles without nucleus or granules recognize opsonins. This recognition is then followed by activation of the phagocytic and the metabolic reactions. Thus, the plasma membrane of these cytoplasts is fully intact, both physically and functionally. PMN cytoplasts, therefore, may be a powerful tool with which to study PMN membrane functions in great detail. Moreover, PMN cytoplasts also provide a unique possibility to study the

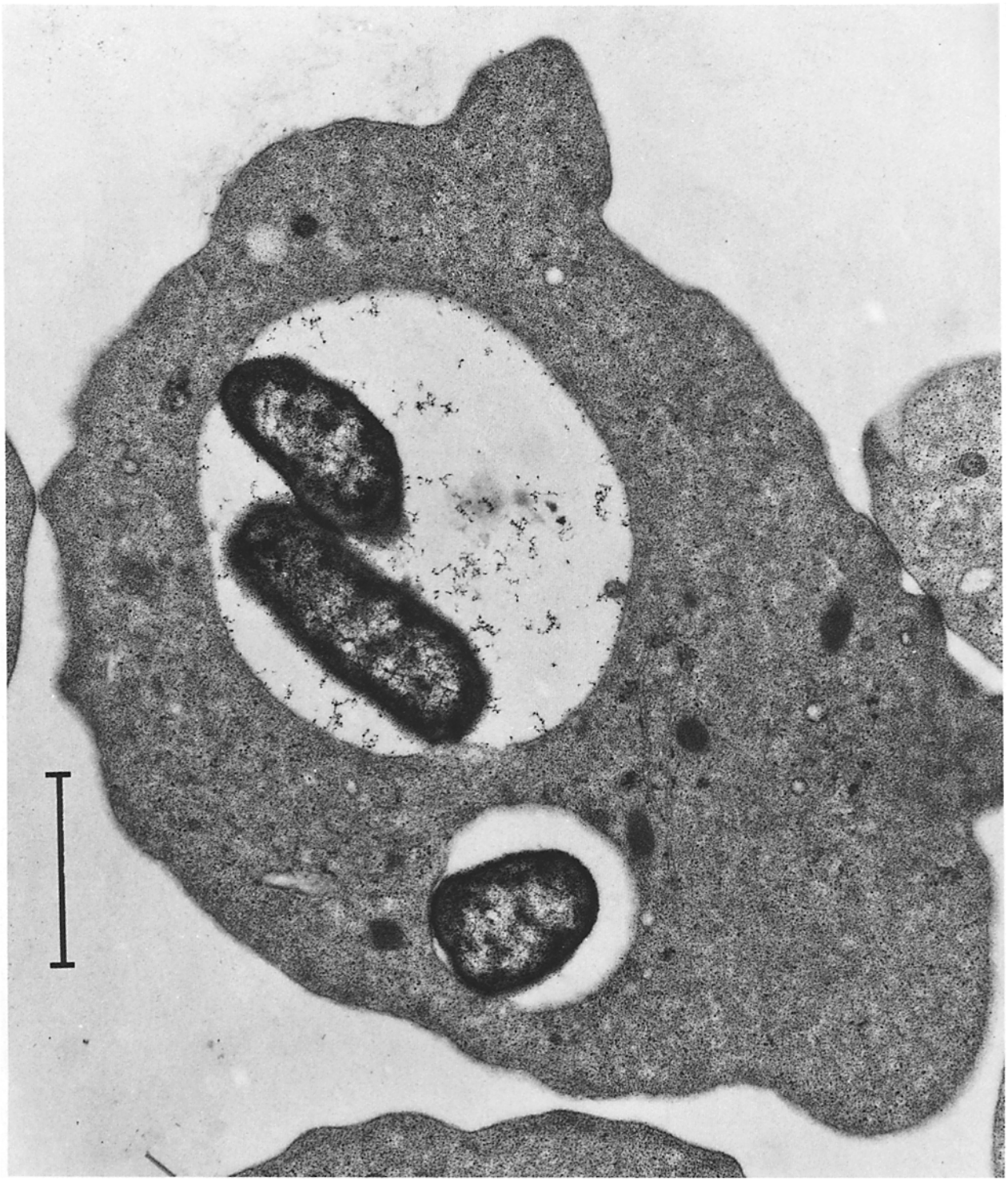

FIGURE 7 Electron micrograph of a PMN cytoplast after incubation with opsonized *E. coli* bacteria. PMN cytoplasts were incubated with *E. coli* bacteria in 10% (vol/vol) human AB serum for 15 min, and fixed and treated as described in the legend to Fig. 1, except that staining for peroxidase activity was omitted. Bar, 1 μm. × 32,000.

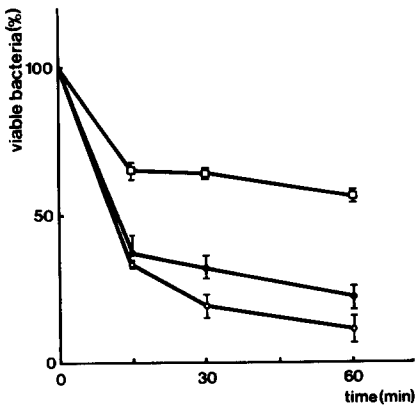

FIGURE 8 Intracellular killing of *S. aureus* by PMN. PMN cytoplasts or the original PMN were mixed with *S. aureus* bacteria in 10% human AB serum and incubated for 7 min at 37°C. The incubations were then centrifuged at 4°C, washed three times with ice-cold medium, and reincubated at 37°C. At the indicated times, the cells were lysed with water; the lysate was diluted with medium, applied to agar plates, and cultured overnight. The number of live bacteria was measured in triplicate by colony counting. Results are expressed as percentage of live bacteria found in the cells before reincubation at 37°C (mean ± SEM of three paired experiments). O, PMN; ●, PMN treated with 12.5% (wt/vol) Ficoll plus 20 μM cytochalasin B for 30 min at 33°C and washed five times; □, PMN cytoplasts.

role of granules and nucleus in PMN functions. A similar reasoning holds true for monocytes, because we found that these cells, too, are enucleated with the technique described in this article (unpublished observations).

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