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Bactericidal Capacity of Phorbol Myristate Acetate-Treated Human Polymorphonuclear Leukocytes

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Thus far, the functional capacity of phorbol myristate acetate- (PMA)-treated human polymorphonuclear leukocytes has been undefined. PMA induced exocytosis of lactoferrin, the specific granule marker, but not of myeloperoxidase, the azurophil granule marker. This phenomenon was demonstrated both biochemically and with fluorescent antibody conjugates. PMA-treated neutrophils contained virtually no specific granules when viewed by electron microscopy. Separation of the granule classes by linear sucrose density gradient centrifugation revealed the loss, from PMA-treated neutrophils, of lactoferrin and the specific granule ($D_{20}^{20} = 1.89$) band usually resolved from normal neutrophils. Cells treated with PMA appeared to retain those functions normally associated with intraleukocytic microbicidal action. The hexose monophosphate shunt activated by phagocytic challenge was present in PMA-treated neutrophils. As demonstrated by electron microscopy, the azurophil granules of these cells appeared intact, and they retained the capacity for degranulation with translocation of myeloperoxidase to the site of phagocytized *Escherichia coli*. The PMA-treated neutrophils also remained capable of degrading the ingested microorganisms. PMA-treated neutrophils exhibited a decrease in phagocytic ability at all levels of bacterial challenge. In the presence of a high multiplicity of bacteria they demonstrated an impairment in killing. These same cells were able to kill low multiplicities of *E. coli* as well as control cells. It thus appeared that the loss of the specific granules, plus other undefined PMA-induced alterations, impaired neither the viability of these neutrophils nor their killing ability in the presence of a modest phagocytic challenge.

Human neutrophilic polymorphonuclear leukocytes (neutrophils) contain two classes of granules, distinguishable by size, density, composition, number, and kinetics of degranulation, that may contribute to their antimicrobial capacities (1-4, 8, 9, 19, 27, 30). The specific or secondary granules, which outnumber the azurophil granules 2 to 1, are formed along the convex face of the Golgi complex during the myelocyte stage of development (2). They are 100 nm in diameter, have a buoyant density of 1.189 (8, 27, 30), and contain, as thus far identified, lactoferrin (LF) and lysozyme, the former protein being unique to the specific granules and presently their only known unambiguous chemical marker (27). The azurophil or primary granules are assembled along the concave face of the Golgi complex during the promyelocyte stage (2). They are 300 nm in diameter, have a buoyant density of 1.20 to 1.22, and contain a panoply of enzymes and other proteins including cationic proteins, proteinases, lysozyme, and myeloperoxidase (MPO). Although both specific and azurophil granules contain substances that have antimicrobial activity in vitro (26), little is actually known of the relative contributions of either granule class or the antimicrobial capacities of the neutrophil. Information on the role of specific granules as mediators of neutrophil antimicrobial action is especially meager.

White and Estensen (11, 31) have reported that phorbol myristate acetate (PMA), a potent co-carcinogen, seems able to induce selective exocytosis of specific granules from human neutrophils. In one report (31) they monitored the exocytosis of specific granule content with alkaline phosphatase, which is not located in these granules (27). In a second publication (11) they used lysozyme as a marker for the specific granules. Although lysozyme is found in the specific granules, it is also compartmentalized within the azurophil granules. It would be difficult to draw

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any unambiguous conclusions about the selective labilization of a granule class, if selective markers for each granule type are not employed. Also using lysozyme as a marker for the specific granules, Wright et al. (32) demonstrated the loss of the specific granule peak from linear sucrose gradients after PMA treatment of human neutrophils. Again the dual localization of lysozyme in both granule types made it difficult to quantitate the PMA-induced loss of specific granules.

With the idea that neutrophils treated with PMA could provide a way to study the relative importance of specific granules to their antimicrobial action, we have now shown that PMA causes neutrophils to expel quantitatively their LF, a specific granule marker, and therefore the matrixes of their specific granules. At the same time PMA leaves the azurophil granule membrane matrixes and MPO intact within the neutrophil. These neutrophils retain the capacity to activate their hexose monophosphate (HMP) shunts and prevent leakage of lactic dehydrogenase from their cytoplasm. They also retain a reduced but discrete capacity to phagocytize and to kill *Escherichia coli*.

The work presented here had three objectives: (i) to examine the PMA-induced selective exocytosis of specific granules of neutrophils with the use of a valid specific granule marker; (ii) to evaluate immunochemically, microscopically, and biochemically azurophil granules and oxidative mechanisms in cells treated with PMA; and (iii) to examine and compare with normal controls phagocytosis and intraleukocytic killing by PMA-treated neutrophils.

(Part of this work was presented at the 13th National Meeting of the Reticuloendothelial Society, New Orleans, La. 15–18 December 1976.)

MATERIALS AND METHODS

Isolation of neutrophils. Four hundred milliliters of normal human blood treated with Plasmagel to remove erythrocytes provided suspensions of neutrophils that were further purified through gradients of 6% Ficoll (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) and 10% hypaque (Winthrop Laboratories, New York, N.Y.) (7). The neutrophils were washed once with medium 199, and the contaminating erythrocytes were removed by hypotonic lysis. Sodium chloride (3.6%) added to the cells restored them to isotonicity. The cell pellets obtained in this manner contained greater than 94% neutrophilic polymorphonuclear leukocytes. Viability was greater than 98%, as determined by trypan blue exclusion.

Preparation of cells in suspension. Purified human polymorphonuclear leukocytes were suspended in medium 199 and added to 50-ml siliconized Erlenmeyer flasks at a concentration of 3×10^7 to 5×10^7/ml in a total volume of 4.5 ml. Human type AB serum was added to a final concentration of 10%.

Preparation of latex beads. Polystyrene latex beads of a uniform 1.1-μm diameter (Dow Chemical Co., Indianapolis, Ind.) were prepared as described elsewhere (18). Immunoglobulin G_3 (gift of James Folds, Department of Bacteriology and Immunology, University of North Carolina School of Medicine, Chapel Hill) was adsorbed to the latex beads by the method of van Oss and Singer (28).

Phagocytosis of latex beads by cells in suspension. Latex beads coated with immunoglobulin G_3 were added to suspended cells at a ratio of 200 beads per cell, and the suspension was incubated with shaking for 60 min at 37°C under 5% CO_2–95% air. Where indicated, PMA was added with the challenge particles. Phagocytosis was terminated by transfer onto ice. The cells were collected by centrifugation at 1,000 rpm for 8 min in a Sorvall SS34 rotor and washed three times to remove uningested latex beads. The extracellular medium obtained from the first centrifugation was further centrifuged at 6,000 rpm for 10 min in an HS rotor to clarify the fluid. The cell-free extracellular medium was transferred to dialysis tubing for concentration by dehydration with Ficoll (Sigma Chemical Co., St. Louis, Mo.). This step was taken to concentrate the soluble proteins for later biochemical and immunological analyses.

Preparation of cells for sucrose density centrifugation and isopycnic centrifugation of neutrophil granules. Purified neutrophils (3×10^7 to 5×10^7/ml) were incubated in suspension for 60 min in medium 199 containing 10% AB serum, in either the presence or absence of PMA (20 ng/ml). At the end of the incubation the cells were washed two times with 0.9% NaCl and suspended in 0.34 M sucrose at a concentration of 3×10^8/ml. The cells were homogenized for 45-s periods at 1,900 rpm in a Potter-Elvejhem homogenizer until 70 to 80% of the cells were ruptured as viewed by phase microscopy. The homogenate was centrifuged at $126 \times g$ for 15 min in an HS rotor in a Sorvall RC2B centrifuge. The $126 \times g$ supernatant was then diluted with 60% sucrose (wt/wt) to achieve a final sucrose concentration of 25% (wt/wt).

A sample of the $126 \times g$ supernatant was layered on top of a 50-ml linear sucrose gradient of 30 to 53% (wt/wt) in SW25.2 tubes. The gradient was centrifuged in a Beckman L2-65B centrifuge at 21°C. The rotor was first rotated for 15 min at 2,000 rpm and then accelerated to 21,000 rpm and run for 2 h (27). The gradients were fractionated with an Isco gradient collector by upward displacement with 60% sucrose (wt/wt), and 1-ml fractions were collected. Turbidity of each fraction was determined by measurement of absorbance at 450 nm. Sucrose concentration was measured with a Zeiss refractometer.

Preparation of cell monolayers. For phagocytosis experiments, purified human polymorphonuclear leukocytes were suspended in medium 199 containing 10% AB serum and added to 60-mm tissue culture dishes at a concentration of 0.5×10^7 to 1×10^7/ml in a total volume of 5 ml. The cells were allowed to adhere for 45 min in a 5% CO_2 chamber (National Appliance Co., Portland, Ore.) at 37°C with gentle swirling every 15 min. For measurements of the HMP shunt, monolayers were prepared in 25-ml Erlenmeyer flasks. Each flask contained 0.5×10^7 to 1×10^7

neutrophils. For the study of fluorescent staining of cells, monolayers were prepared on 18-mm cover slips by the addition of 0.3 ml of a purified neutrophil suspension at a concentration of 3×10^6 to 5×10^6/ml.

Preparation of radiolabeled *E. coli* O111:B4. *E. coli* O111:B4 was grown overnight in Trypticase soy broth at 37°C without shaking. In the morning, 1 ml of the overnight growth medium was inoculated into 40 ml of basal medium (composed of 0.05 M potassium phosphate [pH 7.0], 15 mM $(NH_4)_2SO_4$, 1.7 mM $MgSO_4$, 3.0 μM $FeSO_4$, 0.1% Casamino Acids, and 0.5% D-glucose) containing 20 μCi of *U*-^{14}C-labeled L-amino acid mixture and incubated at 37°C with shaking until log-phase growth was reached (at an absorbance of 0.600 at 650 nm). The bacteria were washed five times with 0.9% NaCl to remove extracellular radioactivity and suspended in medium 199 containing 10% AB serum. Ten million bacteria were equivalent to 2×10^3 cpm. Under the conditions of the study, the bacteria were not sensitive to the bactericidal or lytic actions of serum. Since the bacteria did not replicate during the relevant periods of phagocytosis in control and PMA-treated media, it was possible to compare the phagocytic ability of control and PMA-treated cells.

Measurement of phagocytosis and bacterial killing by cell monolayers. After the preparation of monolayers and two washes with medium, fresh medium was added to the dishes. The dishes were reincubated in the presence or absence of PMA for 60 min at 37°C under 5% CO_2–95% air. At the end of this period the monolayers were again washed two times with medium, and fresh medium was added. Varying amounts of live ^{14}C-labeled *E. coli* O111:B4 were added to the dishes, which were incubated for different periods of time in a rocker platform (Bellco Glass, Inc. Vineland, N.J.) at a setting of 8 under a 5% CO_2 atmosphere at 37°C. Phagocytosis was terminated by the removal of the incubation medium, followed by two washes with phosphate-buffer saline (PBS). The cell pellet was scraped from the dish, suspended in 2.0 ml of sterile deionized water, and sonically disrupted gently for 10 s to release ingested bacteria. This procedure did not affect bacterial viability. Samples were transferred to scintillation vials containing Aquasol-2 (New England Nuclear Corp., Boston, Mass.) and counted in a Beckman LS-250 liquid scintillation spectrometer. Appropriate dilutions were made of the suspended cells, and 0.1-ml samples were plated on Trypticase soy agar. The plates were incubated for 18 h at 37°C before counting. Cells incubated with PMA for 60 min and then reincubated with or without bacteria for an additional 60 min exhibited greater than 97% viability.

To distinguish between actual ingestion and attachment of the bacteria to the cell surface, the following procedure was followed. Cell monolayers were incubated with bacteria at a ratio of 200 bacteria per cell for 60 min under the above conditions. At the end of this period the medium was removed, and the monolayers were washed twice with PBS. The cells were fixed in 1% paraformaldehyde in 0.072 M sodium cacodylate (pH 7.5) for 30 min at 4°C and then stained either wet or dry with anti-*E. coli* O111:B4 serum conjugated to fluorescein isothiocyanate (FITC). Since wet cells are impermeable to the fluorescent conjugate, staining of wet cells reveals only those bacteria that are partially engulfed or attached to the surface of the neutrophil. Staining of dry cells, which have lost their permeability barrier, reveals the total number of cell-associated bacteria. Wet staining revealed no fluorescence, thus indicating that, under these conditions of studying phagocytosis, all the cell-associated bacteria were indeed intracellular and not merely adherent to the cell surface.

HMP shunt study. After the cells had formed a monolayer on the bottom of 25-ml Erlenmeyer flasks as described above, they were prepared for the HMP shunt study. The cell monolayers were washed with medium 199, divided into two sets, and reincubated in the same buffer plus or minus PMA (20 ng/ml) for 60 min under 5% CO_2 at 37°C. At the end of this incubation, the monolayers were washed with PBS to remove the PMA and placed in this buffer for the HMP shunt study. Each flask contained 0.1 ml of 9 mM [1-^{14}C]glucose (10^5 cpm) and 10% AB serum. Heat-killed *E. coli* O111:B4 were added to half the flasks in a ratio of 200 bacteria per cell. PBS was added to a final volume of 1.0 ml, and the flasks were incubated for 60 min at 37°C with gentle shaking. Incubation was terminated by the addition of 0.5 ml of 5% trichloroacetic acid to each flask. Evolved $^{14}CO_2$ was trapped in 0.2 ml of hyamine of hydroxide, contained in a center well.

Antisera. Rabbit antisera to LF and MPO were prepared and rendered monospecific by procedures already described (17, 18, 25a). The antisera were labeled either with FITC (15) (Sigma) or with tetramethylrhodamine isothiocyanate (TRITC) (25) (BBL, Division of Becton, Dickinson and Co., Cockeysville, Md.). Conjugate specificity, dilutions, and fluorochrome-protein ratios are described elsewhere (25a). FITC-labeled antisera to *E. coli* O111:B4 were obtained from Difco Laboratories, Detroit, Mich.

Immunofluorescent staining. The direct method of staining was employed. After incubation the monolayers of cells on 18-mm cover slips were fixed with 1% paraformaldehyde in 0.072 M sodium cacodylate buffer (pH 7.5) for 30 min at 4°C. The cover slips were washed two times with medium and allowed to dry. The cells were then moistened with 0.05 M NaCl in 0.01 M phosphate buffer (pH 7.5) and stained for 30 min at 4°C with labeled conjugate, followed by a 30-min wash with three changes of PBS. The fixation and the drying of the cells make the membrane permeable to the conjugates. Cover slips were mounted in glycerol–PBS (9:1) and viewed on a Leitz-Orthoplan fluorescent microscope with epi-illumination. Controls included blocking of staining with specific antisera or by immunoadsorbance of conjugate by affinity chromatography (25a). Sera obtained from preimmunization bleedings did not stain cells.

Electron microscopy. Neutrophil monolayers incubated with or without PMA (20 ng/ml) for 60 min at 37°C under 5% CO_2–95% air were challenged with heat-killed *E. coli* O111:B4 in a ratio of 100 bacteria per cell for 30 min. Control monolayers were incubated for the same period of time without challenge particles. After incubation the monolayers were washed two times with PBS and fixed for 4 h at 4°C in 1.5% glutaraldehyde in 0.072 M sodium cacodylate-hydrochloride (pH 7.4) containing 0.72% sucrose. After two rinses in 0.158 M tris(hydroxymethyl)aminomethane-

hydrochloride (pH 7.4), the cells were scraped from the dish and stained at room temperature for 1 h with the peroxidase medium of Graham and Karnovsky (12). After three washes in ice-cold 0.158 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.4), the cells were postfixed for 1 h at room temperature in 1.0% osmium tetroxide in Veronal-acetate buffer (pH 7.4) containing 5% sucrose. Postfixation was followed by three rinses in Veronal-acetate buffer (pH 7.4) with 5.71% sucrose. After dehydration through a graded series of ethanol and propylene oxide, the samples were embedded in Luft Epon (21) and allowed to polymerize at 60°C for 3 days. The blocks were sectioned with an LKB Huxley ultramicrotome. Thin sections were stained with saturated uranyl acetate for 20 min and viewed with an AEI EM6B electron microscope.

Preparation of PMA. PMA (Consolidated Midland Corp., Brewster, N.Y.) was dissolved in dimethyl sulfoxide (Sigma) at a concentration of 1 mg/ml and further diluted either with medium 199 or PBS (for the HMP shunt study). The amount of dimethyl sulfoxide (0.002%) present in the incubation did not alter cellular function.

Immunochemical determinations of LF and MPO. LF and MPO were used, respectively, as markers for the specific and azurophil granule classes. With specific antisera and purified LF and MPO as standards, these marker proteins could be quantitated in neutrophil subcellular fractions by single, radial immunodiffusion (22, 24) as described by Mancini et al. (23). The purification of LF and MPO and the preparation of the specific antibodies have been described elsewhere (13, 18, 28).

Chemical and enzymatic determinations. Latex was measured by extraction in *p*-dioxane as described by Werb and Cohn (29). Before extraction, samples were dried overnight at 65°C. Protein was measured by the method of Lowry et al. (20), using lysozyme as the standard. Lactic dehydrogenase, assayed according to Bergmeyer et al. (6), was used to monitor cell viability.

RESULTS

Evaluation of PMA effect on exocytosis by human neutrophils. We employed two approaches to study the effect of PMA on exocytosis by human neutrophils; we examined its effect both on a whole population of neutrophils by biochemical means and at the level of the single cell by immunofluorescence. Disrupted neutrophils can be fractionated on linear sucrose gradients, resulting in the resolution of specific and azurophil granules (8, 27, 30). To quantitate the PMA-induced exocytosis of specific granules, control and PMA-treated neutrophils that were maintained in suspension were homogenized, centrifuged at 1,500 *g* min for 1 min to remove nuclei and unbroken cells, and then centrifuged to equilibrium in linear sucrose density gradients. Turbidimetric measurement of the gradient at 450 nm revealed a specific granule peak in cells incubated without PMA. No such peak was observed in the gradients of PMA-treated cells (Fig. 1). Assay for LF in the gradient of PMA-treated cells demonstrated its absence from the area of the gradient where the specific granules were usually located or elsewhere within the gradient. MPO was present in normal amounts in the azurophil granule peak (Fig. 1).

Although PMA caused a dramatic release of LF from human neutrophils, it did not appear to abolish their phagocytic ability. Microscopic examination of PMA-treated cells incubated in suspension with immunoglobulin G_3-coated latex beads revealed the presence of the beads within these cells. The addition of latex beads in the presence of PMA did not further enhance LF release (Table 1), whereas it did lead to exocytosis of MPO comparable to that normally expected in response to phagocytic challenge in the absence of PMA. Quantitation of the cell-associated latex beads suggested, however, that the PMA might depress the phagocytic capacity of the cells. Control cells contained 1.99 mg of latex per mg of cell protein, whereas PMA-treated cells contained only 0.65 mg of latex per mg of cell protein. Under comparable conditions of phagocytosis, we observed that PMA treatment caused extensive clumping of the suspended cells, as reported by earlier workers (32, 34). Thus, it was not possible to decide whether it was the direct effect of PMA on the cell membrane or the clumping phenomenon that was depressing the ingestion of the challenge particles. To circumvent this problem, all subsequent studies were carried out with neutrophils in monolayers.

Unlike neutrophils treated with PMA in suspension, which exhibited a variable release of LF, neutrophil monolayers (1×10^7 to 2×10^7 cells per dish) incubated with PMA (20 ng/ml) for 60 min released all of their LF as measured by single, radial immunodiffusion. Under these conditions neither MPO nor lactic dehydrogenase was detected in the extracellular medium. The PMA-induced selective release of specific granules from neutrophil monolayers was examined further with immunofluorescent staining and electron microscopy.

Control neutrophil monolayers stained with fluorescent antibody conjugates to LF and MPO revealed the presence of granular staining for both LF and MPO; the staining was evenly distributed in the cytoplasm surrounding the nucleus (Fig. 2A and B). PMA-treated cells retained the capacity to bind anti-MPO conjugates, but staining for LF was either absent or greatly diminished (Fig. 2C and D). Comparison of control and PMA-treated neutrophils by elec-

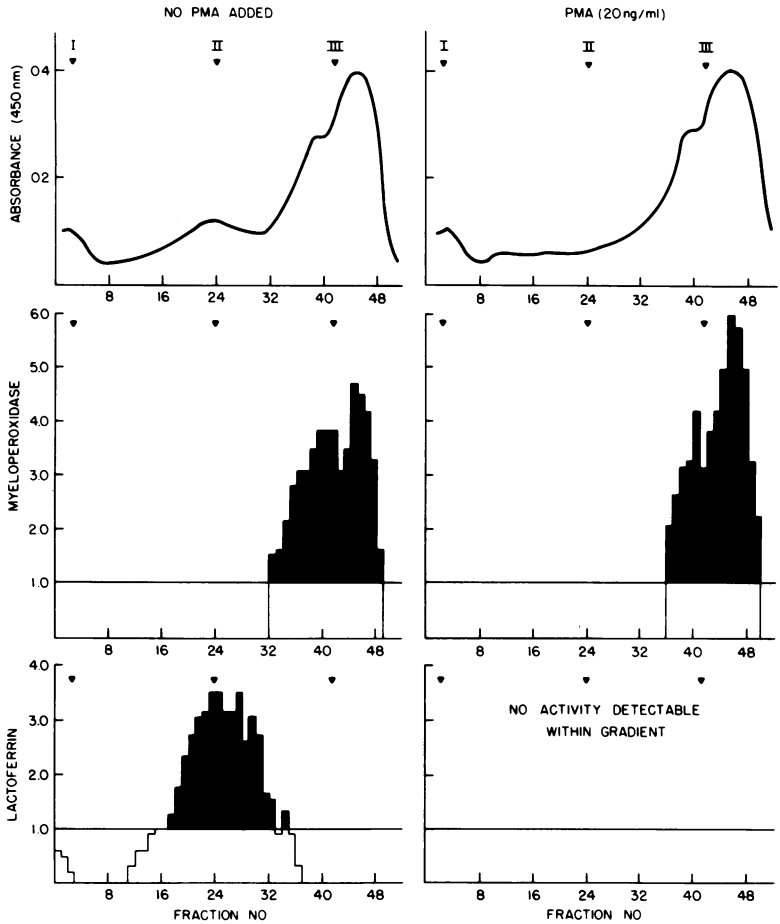


FIG. 1. *Isopycnic centrifugation of cytoplasmic granules from control and PMA-treated neutrophils. Neutrophils were incubated in suspension for 60 min in the presence or absence of PMA (20 ng/ml). After two washes with 0.9% NaCl, the cells were suspended in 0.34 M sucrose at a concentration of 3 × 10^8/ml and homogenized (see the text). The homogenate was centrifuged at 126 × g for 15 min, and the 126 × g supernatant was layered on top of a 30 to 53% (wt/wt) linear sucrose gradient. Isopycnic centrifugation was carried out as described in the text. Turbidity of the gradient fractions was measured at 450 nm. The scale of the ordinate for LF and MPO was calculated by the method of Beaufay et al. (5). A line is drawn across each histogram and corresponds to 1 on the ordinate. The bars are blackened where they extend above the line to indicate that the constituent has been concentrated in that fraction. The numbers on the abscissa indicate the number of fractions collected from the gradients. The granules sedimented from left to right. LF and MPO were measured by single radial immunodiffusion. Recoveries from the gradient were 90% for MPO and 100% for LF: control MPO = 411 μg (input = 456 μg); PMA − MPO = 354 μg (input = 393 μg); control LF = 1,444 μg (input = 1,445 μg). In this particular study, PMA caused the extracellular release of 92% of the total cellular LF. The postnuclear supernatant that was placed over the gradient contained a total of 60 μg of LF. The sensitivity of the assay is 6 μg of LF per ml. By the time the material was distributed within the gradient, the amount of LF found in each fraction was below the sensitivity of the assay. (I) Soluble protein and membranous fraction; (II) specific granule peak; (III) azurophil granule peak.*

tron microscopy revealed the loss of specific granules from PMA-treated cells, with retention of the peroxidase-positive azurophil granules (Fig. 3A and B).

Effect of PMA on the oxidative metabolism of neutrophil monolayers. It is believed that for MPO to exercise its antimicrobial action, H_2O_2 and Cl^- must be present (16). Either the activation of the HMP shunt is a reflection of increased H_2O_2 production, or it may provide reduced nicotinamide adenine dinucleotide phosphate, which is believed to be needed for

TABLE 1. *Effect of PMA and phagocytosis on exocytosis by human polymorphonuclear leukocytes in suspension*

Marker	Activity (%) recovered in the extracellular medium[a]			
	Cells − PMA		Cells + PMA	
	Resting	Phagocytizing	Resting	Phagocytizing
LDH[b]	1.3 ± 0.2 (5)	2.2 ± 1.0 (5)	1.9 ± 0.4 (3)	2.5 ± 0.2 (3)
LF	6.7 ± 0.4 (5)	46.1 ± 0.8 (4)	79.4 ± 1.7 (5)	76.2 ± 1.0 (3)
MPO	0 (5)	16.7 ± 0.5 (4)	0 (3)	18.5 ± 0.3 (3)

[a] Cells were incubated without or with PMA at a concentration of 20 ng/ml. The activity found in the extracellular medium is expressed as percentage of the total activity measured in the whole cell prior to incubation. A sample of 2.4×10^8 neutrophils contains 1.2×10^3 μg of LF and 4.7×10^2 μg of MPO. The cells (4.7×10^7/ml in a total volume of 5 ml) were incubated in suspension in medium 199 (containing 10% AB serum) for 60 min at 37°C with shaking under 5% CO_2. The data are presented as the mean ± standard error, with the number of determinations indicated in parentheses. The challenge particles were polystyrene latex beads (1.1-μm diameter) coated with immunoglobulin G_3. Beads were added to the cells in a ratio of 200 beads per cell.

[b] LDH, Lactic dehydrogenase.

H_2O_2 production (33). To determine if the HMP shunt was active in PMA-treated cells and thus to obtain an indication of the oxidative metabolism of the PMA-treated neutrophils, we compared the ability of control and PMA-treated cells to increase their HMP shunt activity under different conditions. In the presence of challenge particles, control cells exhibited, as expected, a marked stimulation of shunt activity (Table 2). Unchallenged PMA-treated cells also exhibited a burst of shunt activity. Cells first treated with PMA, then washed and incubated with [1-^{14}C]-glucose also showed activation above control values. In the presence of challenge particles, these cells exhibited an additional increase in shunt activity.

Phagocytosis, degranulation, and bactericidal capacity of PMA-treated neutrophil monolayers. To examine the effect of PMA on the phagocytic capacities of neutrophil monolayers, radiolabeled log-phase *E. coli* O111:B4 were added to the monolayers at different ratios of bacteria to cell. At all levels of bacteria added, the PMA-treated cells exhibited a reduced phagocytic capacity as compared with control cells (Table 3; Fig. 4A). With a high multiplicity of bacteria (200 bacteria to 1 cell), ingestion then was examined as a function of time. Cells treated with PMA exhibited an initial rate of ingestion that was half that of normal cells (Fig. 4B).

Examination by electron microscopy of the postphagocytic neutrophil monolayers revealed the presence of bacteria completely enclosed within phagocytic vacuoles in PMA-treated cells (Fig. 3C). The electron micrographs also revealed that the azurophil granules of the PMA-treated cells retained the capacity to fuse with and to discharge their contents into the phagosomes (Fig. 3C). Extensive degradation of the ingested bacteria also was apparent in PMA-treated neutrophils.

When challenged with a low dose of bacteria, the PMA-treated cells appeared to be as efficient as the controls in killing the ingested bacteria (Table 3). At a high multiplicity of bacteria, these cells exhibited a definite impairment in killing as compared with control cells.

DISCUSSION

Our initial aim was to use PMA as a tool to obtain human neutrophils selectively deficient in specific granules and to study their subsequent microbicidal capacity. The ability of PMA to cause the selective exocytosis of specific granules from human neutrophils is well documented (11, 31, 32), although valid specific granule markers were not used in these earlier studies. The first part of this study was concerned with the reexamination of this observation. Using unambiguous markers for the specific and azurophil granules, LF and MPO, respectively, we demonstrated under our conditions that neutrophils can be induced to expel quantitatively their specific granules; the azurophil granules remain morphologically and functionally unaffected by PMA. Centrifugation of PMA-treated neutrophil homogenates in a linear sucrose density gradient revealed the loss of specific granules with the quantitative preservation of the azurophil granules at their normal density (Fig. 1). Immunofluorescence and electron microscopy also confirmed the quantitative loss of specific granules from all PMA-treated cells, with retention of azurophil granules (Fig. 2 and 3).

In addition to the loss of specific granules, we wondered if PMA affected those functions well

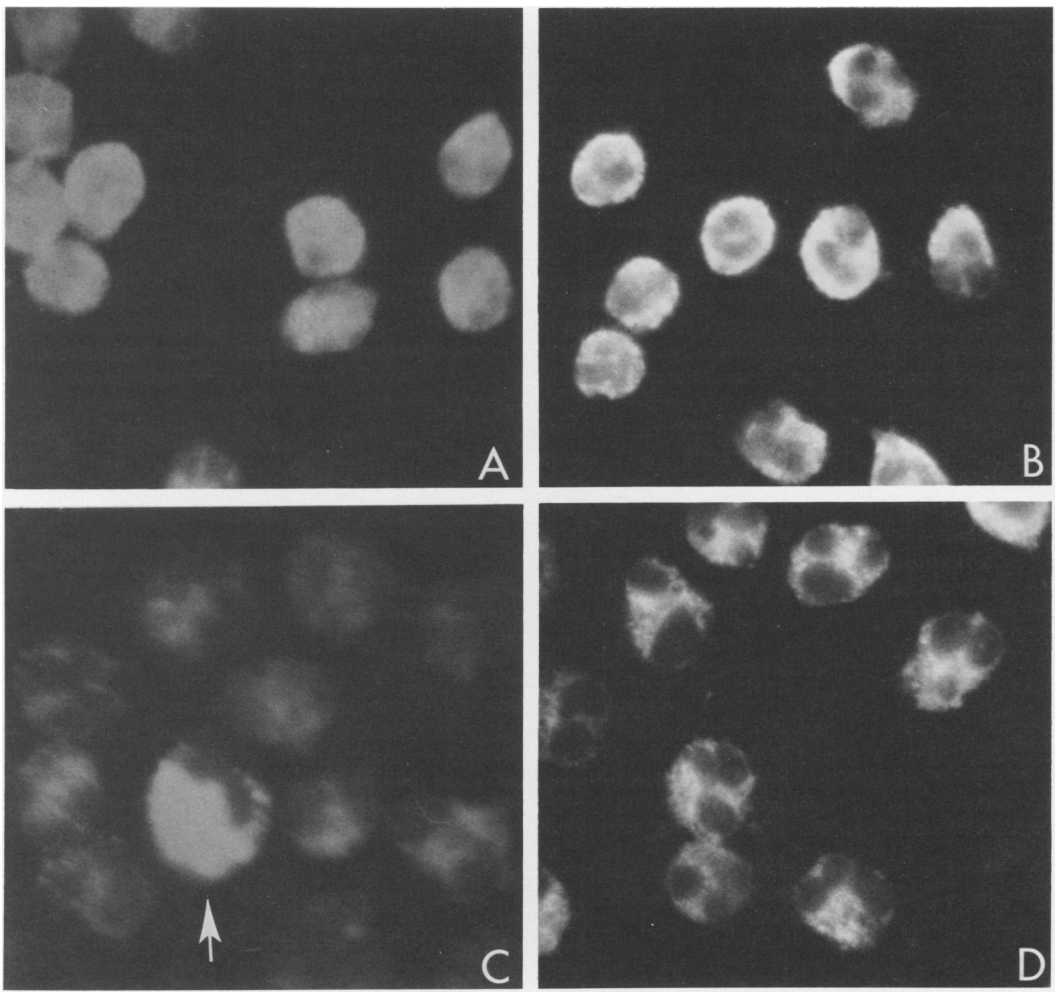
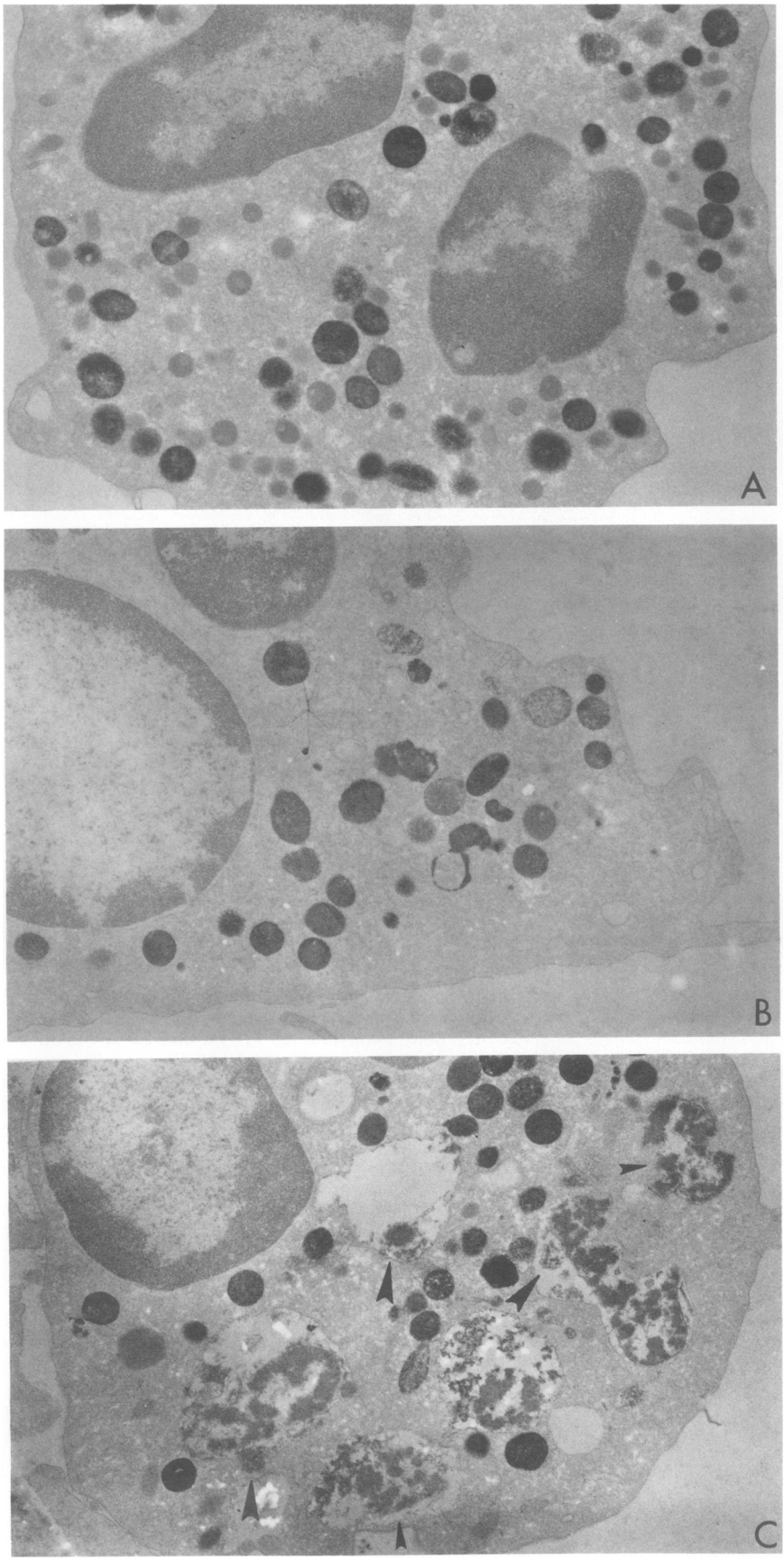


FIG. 2. *Measurement of LF and MPO with fluorescent antibody conjugates in control and PMA-treated neutrophil monolayers. (A) TRITC anti-LF conjugate with control cells; exposure time, 2 min 28 s. (B) FITC anti-MPO conjugate with control cells; exposure time, 2 min 40 s. (C) TRITC anti-LF conjugate with PMA-treated cells; exposure time, 13 min. (D) FITC anti-MPO conjugate with PMA-treated cells; exposure time, 2 min 45 s. The magnification is ×840. Cells were stained for LF and MPO after a 60-min incubation with or without PMA (20 ng/ml). Note that the exposure time for the anti-LF-PMA-treated cells (C) was 5 times longer than for the control (A). If the same exposure time had been used, the PMA-treated cells would not have been visible. (C) contains an autofluorescent eosinophil (arrow). PMA treatment causes the individual cells to spread more on the glass surface, and the granules in turn are more readily seen as individual points of light. Thus, the staining for MPO in control cells (B) appears to be brighter than in the PMA-treated cells (D), because the fluorochromes are closer together. The photographs of the FITC conjugates are relatively brighter than those of the TRITC conjugates, because the black and white photographic emulsion is more sensitive to the apple-green hue of the FITC than to the orange-red hue of the TRITC.*

documented to be associated with intraleukocytic microbicidal activity (16, 33). The presence of PMA during incubation of neutrophils with latex beads did not alter the degranulation of MPO from azurophil granules (Table 1). It has been reported that PMA alone can activate the HMP shunt (10). We found that the degree of activation could be enhanced further by phagocytic challenge (Table 2). We also showed by electron microscopy that in PMA-treated neutrophils presented with phagocytic challenge MPO was translocated from the azurophil gran-


A
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ules to the site of the phagocytized *E. coli* (Fig. 3C). Thus the HMP shunt, the azurophil granules, their MPO, and the capacity for degranulation and translocation of the MPO were present in cells treated with PMA.

The last part of the study focused upon the ability of the PMA-treated neutrophils to ingest and to kill *E. coli* O111:B4. At all multiplicities of bacteria the PMA-treated neutrophil monolayers exhibited a reduced phagocytic capacity (Table 3). A similar observation had been made with cells in suspension (10). As demonstrated with model membranes, the lipophilic nature of PMA allows it to intercalate into the plane of the membrane (14). The insertion of PMA into the membrane might interfere with or alter the receptors involved in the attachment of bacteria to the cell surface, thus resulting in reduced ingestion by the neutrophil.

In conjunction with an overall reduction in phagocytosis, these cells exhibited a decrease in the rate of ingestion (Fig. 4B). Despite a reduced phagocytic capacity, the PMA-treated cells retained full killing capacity at low multiplicities of bacteria (Table 3). Only in the presence of a large number of bacteria did they exhibit a definite impairment in killing as compared with their control cells. Thus, it appears from this study that the specific granules contribute neither to the basic maintenance of cellular integrity and viability nor to the vital antimicrobial mechanisms in the defense of the cells against a low dose of bacteria.

TABLE 2. *Activation of the HMP shunt by phagocytizing control and PMA-treated neutrophils*

Condition	Counts per minute per 2×10^6 neutrophils[a]
Resting—control	313 ± 9
Phagocytizing—control	1,197 ± 94
Resting—post-PMA-treated	1,645 ± 42
Phagocytizing—post-PMA-treated	2,143 ± 57

[a] The number of neutrophils was derived from the amount of protein determined in each flask. After the preparation of monolayers, the cells were washed, fresh medium was added, and the flasks were reincubated for 60 min without or with PMA (20 ng/ml). After this second incubation the cells were washed with PBS, and HMP shunt activity was measured under resting and phagocytizing conditions (see the text). The counts per minute indicate the amount of $^{14}CO_2$ liberated from [1-^{14}C]glucose during a 60-min incubation. The data are expressed as the mean of three determinations ± standard error of the mean. The data are representative of three different experiments.

TABLE 3. *Phagocytic and bactericidal capacity of PMA-treated neutrophils*[a]

Neutrophils	Ratio of bacteria to cell[b]	% Ingested[c]	% Killed[d]
Experiment 1			
Control	1:1	45.6 ± 1.3	100
	10:1	56.2 ± 2.3	99.3
	200:1	14.6 ± 9.8	58.2
PMA-treated	1:1	26.2 ± 0.7	97.8
	10:1	23.9 ± 0.9	97.4
	200:1	11.4 ± 0.3	6.0
Experiment 2			
Control	1:1	43.8 ± 1.5	100
	10:1	28.1 ± 1.1	99.7
	200:1	11.2 ± 0.8	90.4
PMA-treated	1:1	16.4 ± 0.7	95.0
	10:1	17.1 ± 0.8	93.5
	200:1	6.3 ± 0.2	52.9

[a] Neutrophil monolayers (1.2×10^7 cells per dish) were incubated with or without PMA (20 ng/ml) for 60 min, washed, and then challenged with radiolabeled *E. coli* O111:B4. Two experiments are presented to demonstrate individual variations observed in apparently normal donors. Normal killing at a low ratio of bacteria to cell and defective killing at a high ratio of bacteria to cell were obtained for PMA-treated neutrophils in six different experiments. The data are expressed as the mean ± standard error of three determinations.

[b] Bacteria input: 1:1 = 1.95×10^7; 10:1 = 1.95×10^8; 200:1 = 4.00×10^9.

[c] Ingestion was measured by the amount of cell-associated radioactivity, which in turn was equivalent to a certain number of colonies.

[d] Viability of cell bacteria was determined by plating samples of ruptured neutrophil suspensions (see the text). The microbicidal capacity of the neutrophils (percentage of killing) was calculated by dividing the number of viable colonies by the total number of bacteria that were ingested, and subtracting the resulting percentage from 100%.

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FIG. 3. *Demonstration of phagocytosis and degranulation by electron microscopy. Control and PMA-treated neutrophil monolayers were incubated with or without heat-killed E. coli O111:B4 for 30 min. The cells then were processed under identical conditions as described in the text. (A) Unchallenged control cell. (B) Unchallenged PMA-treated cell; note that the specific granules are absent, and peroxidase-positive azurophil granules are present. (C) PMA-treated cell after 30 min of incubation with bacteria; note the presence of peroxidase-positive phagocytic vacuoles (large arrowhead) and the extensive degradation of bacteria (small arrowhead). Magnification is ×17,000.*

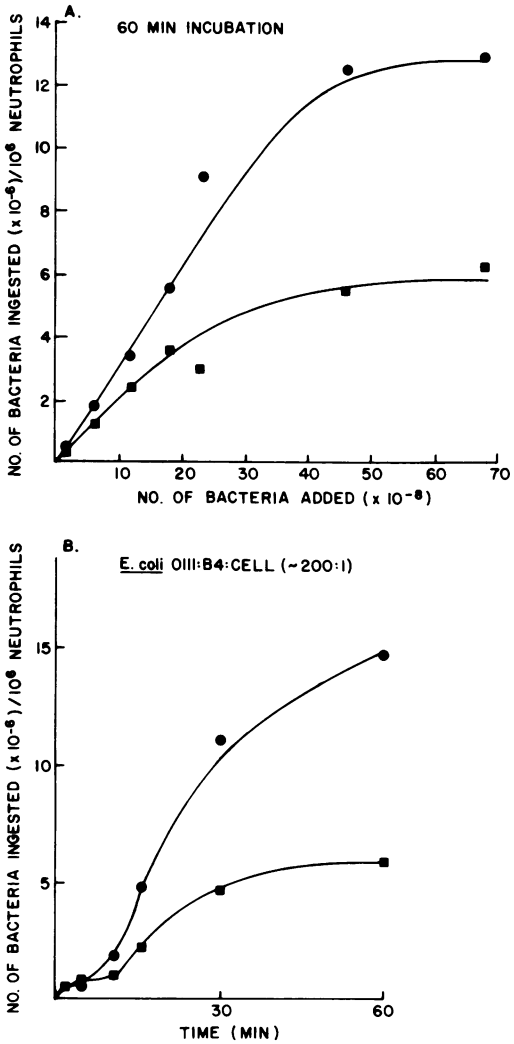

FIG. 4. *(A) Ingestion by neutrophils as a function of increasing number of bacteria. Cell monolayers in medium 199 (containing 10% AB serum) were incubated in a rocker platform at 37°C under 5% CO_2 with varying concentrations of bacteria for 60 min after previous incubation with or without PMA (20 ng/ml) for 60 min. The number of adherent neutrophils ranged from 1 × 10^7 to 2 × 10^7 per dish. (B) Ingestion by neutrophils as a function of time. Radiolabeled E. coli O111:B4 were added to cell monolayers at a ratio of ~200 bacteria per cell after previous incubation with or without PMA (20 ng/ml for 60 min). The number of adherent neutrophils ranged from 1 × 10^7 to 2 × 10^7 per dish. This experiment is representative of four different experiments. Symbols:* (●) *Control neutrophils;* (■) *PMA-treated neutrophils.*

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