Selective Labilization of Specific Granules in Polymorphonuclear Leukocytes by Phorbol Myristate Acetate

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The action of phorbol myristate acetate (PMA), the active principle of croton oil, on polymorphonuclear leukocytes (PMNs) has been evaluated in this study. Small amounts of PMA caused the rapid development of vacuoles in neutrophils and the disappearance of specific granules. Histochemical and cytochemical studies revealed that alkaline phosphatase activity was transferred to vacuoles and disappeared from the cells, while myeloperoxidase activity remained associated with intact azurophilic lysosomes. Electron-dense tracers indicated that the vacuole membranes originated, at least in part, from the cell wall of the neutrophils. The results indicate that PMA stimulates events remarkably similar to those which take place when bacteria are engulfed by PMNs, except for the failure of azurophilic lysosomes to participate in PMA-induced vacuole formation. PMA appears to be the first chemical agent capable of inducing selective labilization of specific granules in the neutrophil (Am J Pathol 75:45–60, 1974).

PHAGOCYTOSIS BY POLYMORPHONUCLEAR LEUKOCYTES (PMNs) involves the incorporation of foreign particles into surface membrane invaginations which amputate their connection to the cell wall and migrate into the cytoplasm as isolated vacuoles.[1] Subsequently, storage granules dispersed throughout the cytoplasm fuse with the membrane of the phagocytic vacuole and empty their contents around ingested matter.[1,2] Two different types of storage organelles, the primary or azurophilic granules and the secondary or specific granules, are involved in the process of degranulation following uptake of foreign substances into PMNs.[3] Primary granules are lysosomes containing a variety of hydrolytic enzymes including myeloperoxidase.[4] Specific granules are rich in cationic proteins, lysozyme and alkaline phosphatase. Recently, it has been suggested that specific granules may interact with phagocytic vacuoles before azurophilic lysosomes.[5] The basis for the differential degranulation has not been defined.

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We have recently pursued a number of investigations into the action of phorbol myristate acetate (PMA), the active principle of croton oil, on blood cell structure and function.[6-8] Zucker, Kim, Belman and Troll [9] demonstrated that PMA is a potent stimulus of platelet aggregation, and our studies indicated that the agent causes selective labilization of platelet storage organelles. In the course of these experiments we observed an unusual effect of PMA on PMNs. The drug caused selective disappearance of specific granules in neutrophils without lysing azurophilic lysosomes or destroying the integrity of the cells.

Materials and Methods

General

The procedures used to obtain blood from normal adult donors, mix the samples with 40 to 50 units of heparin/10 ml of blood; separate white-cell-rich plasma by spontaneous sedimentation for 1 to 2 hours at room temperature, wash and resuspend the cells in Hank's buffered salt solution (HBSS), pH 7.3; and prepare control and experimental leukocyte suspensions for study in the electron microscope were described in detail in recent publications.[9-13] Phorbol myristate acetate (12-*O*-tetradecanoyl-phorbol-13-acetate, PMA) was dissolved in dimethylsulfoxide at a concentration of 1 mg/ml and kept frozen between experiments.[7,8] Just before use the agent was diluted in acetic acid–sodium acetate buffer, pH 6.5, to the desired final concentration.

PMA was added in 0.1-ml volumes to 0.9-ml samples of washed leukocytes. The cells were exposed to PMA at final concentrations of 1, 10 and 100 ng, and 1 to 10 μg/ml for intervals of 5, 10, 15, 30 and 60 minutes at 37 C. In some experiments the samples were tumbled during incubation, while in the majority they were maintained in suspension in a constant temperature waterbath. Control samples of washed leukocytes were combined with buffer and incubated for similar periods of time. Experimental and control samples were fixed in suspension with an equal volume of 0.1% glutaraldehyde in White's saline. After 15 minutes, the cells were sedimented to buttons, the supernatant decanted, and fresh 3% glutaraldehyde in White's saline added to the pellets. After exposure to glutaraldehyde for 60 minutes the cells were sedimented, washed in buffer, and refixed for 1½ hours in 1% osmic acid. All samples were dehydrated in a graded series of alcohols and embedded in Epon 812. Thin sections were usually stained with uranyl actetate and lead citrate to enhance contrast, and examined in a Philips 200 electron microscope.

Special Studies

Cytochemical localization of endogenous myeloperoxidase activity was carried out according to a slight modification of the method of Graham and Karnovsky.[14,15] Washed leukocytes with or without PMA were fixed initially in glutaraldehyde or glutaraldehyde-paraformaldehyde, washed several times in buffer, incubated for 1 hour in medium containing diaminobenzidine, hydrogen peroxide and dimethylsulfoxide, and refixed in osmic acid. Peroxidase reaction product produced by the activity of endogenous enzyme was readily visible in the electron microscope. Alkaline phosphatase activity was demonstrated histochemically [16,17] on smears made from leukocytes incubated for 15 minutes with various concentrations of PMA or buffer

and examined for localization and intensity of reaction product in the light microscope.

Vital and nonvital electron-dense stains were used to study changes in the conformation and integrity of leukocyte membranes after exposure to PMA.[18,19] Thorium was added to leukocyte samples 5 minutes before PMA, 5 minutes after the agent, or at the same time as the drug. Lanthanum nitrate was combined with osmic acid and used as a passive stain during the third step of fixation for PMA-treated leukocytes. Some leukocyte samples exposed to 100 ng or 1 μg of PMA for 5 minutes were prepared for study in the electron microscope by the freeze-etch technic in the same manner used earlier for blood platelets.[20]

Results

Normal Leukocyte Morphology

The fine structure of human leukocytes observed in thin sections of glutaraldehyde–osmic acid-fixed, plastic-embedded cells obtained from peripheral blood was described in previous publications.[21] Vacuoles of various sizes are occasionally present in the cytoplasm of neutrophils and other white blood cells, but they are seldom a dominant feature of normal leukocyte morphology.

Morphology of PMA-Treated Leukocytes

General Effects of PMA

Intracellular alterations were evident in the cytoplasm of neutrophils within a short time after exposure to PMA. The characteristic changes were always present after 5 minutes in cells combined with 100 ng or more of PMA, frequent in leukocytes exposed to 10 to 20 ng, and absent in neutrophils incubated with a final concentration of 1 ng/ml. At 30 or 60 minutes of incubation the changes produced by 10 to 20 ng or greater were evident in every cell, and rare cells combined with 1 ng/ml revealed some alterations.

Specific Effects of PMA

Neutrophils exposed to 10 to 100 ng of PMA for 5 minutes or longer developed a marked increase in the number of intracellular vacuoles (Figure 1). The vacuoles present in neutrophils exposed to 100 ng or more were larger in size and more abundant than in cells exposed to lower concentrations, but by 30 to 60 minutes the difference was minimal. The changes which developed in tumbled leukocytes were nearly identical to those in unstirred samples of white cells maintained at 37 C, but vacuoles developed more rapidly in stirred neutrophils and the cells tended to form aggregates.

Formation of vacuoles and increase in their size and number in PMA-treated neutrophils was associated with a decrease in the number

of granules present in the cytoplasm. Specific granules appeared to be more affected by the development of intracellular vacuoles than azurophilic lysosomes, but this could not be stated with confidence on the basis of morphology alone. Other structures including the cell surface and nucleus did not appear to be significantly altered by PMA.

Cytochemical and Histochemical Studies of PMA-Treated Neutrophils

The localization of endogenous myeloperoxidase was examined in neutrophils 5 to 15 minutes after exposure to final concentrations of 1, 10 and 100 ng and 1 μg of PMA (Figure 2 and 3). Enzyme reaction product was regularly confined to normal-sized azurophilic granules in the cytoplasm of control and PMA treated neutrophils. Formation of intracellular vacuoles did not appear to affect the reaction of azurophilic granules, although they were generally crowded aside by expanding vacuoles. At concentrations of 100 ng or higher an occasional vacuoles present in neutrophils incubated 15 minutes contained the reaction product of peroxidase activity, but this was the exception rather than the rule.

Light microscopy of PMA-treated neutrophils stained for alkaline phosphatase activity revealed a sequence almost the reverse of that observed in the electron microscopic evaluation of myeloperoxidase. Control cells incubated for alkaline phosphatase revealed an intense particulate stain in their cytoplasm. Neutrophils incubated with 10 to 20 ng of PMA for 15 minutes before enzyme staining revealed a less intense reaction for alkaline phosphatase than in control preparations. The reaction product in neutrophils exposed to 100 ng or more of PMA was variable. Some cells with large intracellular vacuoles contained no visible stain. In other neutrophils the alkaline phosphatase activity was associated with a few cytoplasmic granules and smaller vacuoles. The decrease in staining suggested that the increase in the number and size of vacuoles eventually resulted in the loss of alkaline phosphatase reactivity in the light microscope.

Localization of Vital and Passive Electron-Dense Stains

Leukocytes combined with thorium dioxide before or simultaneously with addition of PMA revealed particulate stain on the cell surfaces and within the cytoplasmic vacuoles. Control cells also took up thorium dioxide (Figure 4), but the stain particles were confined to small vesicles. In some PMA-treated cells the thorium dioxide appeared to be in the process of incorporation into large invaginations of the surface membrane (Figures 5 and 6).

The appearance of leukocytes treated with PMA and exposed to lanthanum nitrate during the third step of fixation did not differ significantly from control cells. Electron-dense stain covered the surface of the cells but did not penetrate into vacuoles filling the cytoplasm of PMA-treated neutrophils (Figures 7 and 8). Some vacuoles close to cell surfaces, however, were lined with the dense tracer.

Freeze-Cleaved, PMA-Treated Neutrophils

To determine if PMA produced specific changes in the structure of leukocyte membranes, samples of cells incubated for 5 minutes with 1 μg or 100 ng of PMA were prepared for study in the electron microscope by the freeze-fracture technic. Examination of the replicas revealed bubble-like protuberances or evacuations in the cytoplasm comparable to the vacuoles evident in thin sections (Figure 9). The surface granularity of the vacuoles and cell membranes of freeze-cleaved, PMA-treated cells did not differ from normal leukocytes. The number and organization of intercalated particles within the bilayer of neutrophil surface and granule membranes were not significantly altered by the concentrations of PMA used in this study.

Effects of PMA on Other Cells in Circulating Blood

The leukocyte samples contained significant numbers of other types of cells found in blood. As a result the influence of PMA on different cell types could be compared directly with the response of neutrophils in the same sample (Figure 10). Platelets appeared to be the most sensitive to the action of PMA, while erythrocytes were unaffected by the concentrations used in this study. Eosinophils occasionally contained a vacuole when neutrophils were filled with them. Monocytes seemed to be as sensitive as neutrophils to PMA, but did not develop as many vacuoles in their cytoplasm. Lymphocytes remained unaffected over the period of incubation employed in this study.

Discussion

The findings of this study indicate that small quantities of phorbol myristate acetate (PMA) can cause nearly selective disappearance of specific granules from PMNs *in vitro*. Vacuole formation accompanied the loss of specific granules, and the number and size of the empty membrane-bound organelles depended on the concentration of PMA and the length of the incubation period. Azurophilic granules in PMA-treated neutrophils were virtually unaffected by the drug. High concentrations of the agent and prolonged exposure resulted in damage

to some azurophilic granules, but their lysis appeared secondary to the massive vacuole formation and labilization of specific granules.

Cytochemical and histochemical findings also suggest that PMA selectively labilized specific granules. The reaction product of myeloperoxidase activity was localized to intact azurophilic granules and seldom appeared in the swollen vacuoles of PMA-treated neutrophils. Alkaline phosphatase activity declined in neutrophils after exposure to PMA. The reaction product shifted from granules to small vacuoles, and disappeared in PMNs which had formed multiple large cytoplasmic vacuoles. Persistence of the azurophilic lysosomes containing myeloperoxidase and translocation and disappearance of the alkaline phosphatase restricted to specific granules support the suggestion that the action of PMA is focused on the secondary organelles.

The simultaneous development of vacuoles and disappearance of specific granules in PMA-treated neutrophils suggested that membranes of the empty sacs might form by fusion of membranes remaining after labilization of the storage organelles. If this were the case, then the newly formed vacuoles and their contents would be isolated from the cytoplasm and the exterior of the neutrophil. However, the loss of alkaline phosphatase reactivity on histochemical staining of vacuolated cells raised the possibility that vacuoles might have a connection to the surrounding plasma at some stage in their evolution. If vacuoles were formed in part from the surface membrane of the PMA-treated neutrophil, as well as from labilized specific granules, then a route would be available for discharge of alkaline phosphatase and other substances stored in these organelles without simultaneous loss of cytoplasmic enzymes suggestive of cell damage. This hypothesis was tested in the present investigation through the use of electron-dense tracers. Thorium dioxide was employed as a vital stain by combining it with leukocytes before or at the same time as PMA. Dense particles of the stain were present on cell surfaces and in most of the newly formed vacuoles. Control cells also took up thorium dioxide, but the stain was confined to a few small vesicles. Lanthanum nitrate employed as a passive stain coated the surface of PMA-treated cells and entered a few vacuoles near the cell periphery. The majority of vacuoles deep in the cytoplasm were not stained by lanthanum. Inclusion of thorium particles in nearly every vacuole in PMA-treated neutrophils and the exclusion of lanthanum indicate that the vacuoles form at least in part by invagination of the cell surface and eventually seal off the connection as they migrate into the cytoplasm.

Disappearance of specific granules in PMA-treated neutrophils prob-

ably results from interaction with the invaginated cell surface. The events are remarkably similar to the formation of phagocytic vacuoles during uptake of bacteria by neutrophils and the subsequent fusion and degranulation of storage organelles.[1,2] The difference is that both azurophilic lysosomes and specific granules fuse with and discharge into phagocytic vacuoles,[3] whereas the specific granules are selectively involved in the interaction with the invaginated surface of PMA treated neutrophils. Failure of azurophilic granules to react with the cell surface or vacuoles in PMA-treated neutrophils may be related to the absence of organisms or other particulates in the newly formed sacs.

The disappearance of alkaline phosphatase from the PMA-treated neutrophils evaluated in this study by histochemical methods [16,17] is supported by biochemical analysis which will be reported separately.[22] Preliminary observations indicate that lysozyme and alkaline phosphatase increase rapidly in the supernatant after treatment of neutrophils with PMA, while the cytoplasmic enzyme, glucose-6-phosphate dehydrogenase, and the enzymes of azurophilic granules, myeloperoxidase and β-glucuronidase, remain associated with the vacuolated PMNs. In another study we have found that PMA stimulates the same burst of oxygen consumption by neutrophils that results from the uptake of bacteria or other particulates.[23] The accumulated experimental observations indicate that PMA is an extremely important chemical agent for evaluating basic mechanisms involved in phagocytosis, degranulation, and the triggering of metabolic processes in PMNs without using particulate material.

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Acknowledgments

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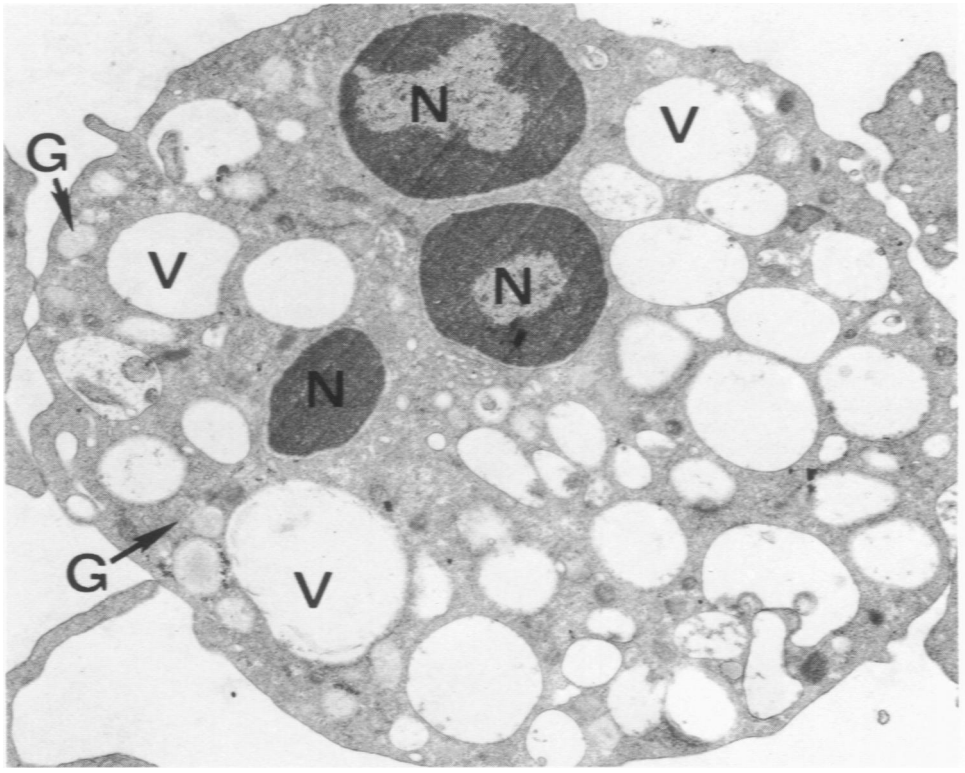
[*Illustrations follow*]

Legends for Figures

Fig 1—Polymorphonuclear leukocyte (PMN) from a sample incubated for 15 minutes with phorbol myristate acetate (PMA) at a final concentration of 1 μg/ml. Nuclear lobes (*N*) and the cell surface appear undamaged. A few storage granules (G) are present in the cytoplasm, but most have disappeared. The cytoplasm is filled with swollen vacuoles (*V*) of various sizes. Development of vacuoles and disappearance of storage organelles are the characteristic morphologic effects resulting from the action of PMA on neutrophils (× 16,000).

Fig 2—Neutrophil from a leukocyte sample incubated for 10 minutes with PMA at a final concentration of 100 ng/ml, then fixed and incubated for the cytochemical demonstration of myeloperoxidase. Electron-dense reaction product of myeloperoxidase activity is confined to intact azurophilic lysosomes in the cell cytoplasm. Vacuoles (*V*) formed in the cell after exposure to PMA do not contain reaction product. The nucleus (*N*) appears undamaged. The section was not stained with uranyl acetate or lead citrate (× 14,300).



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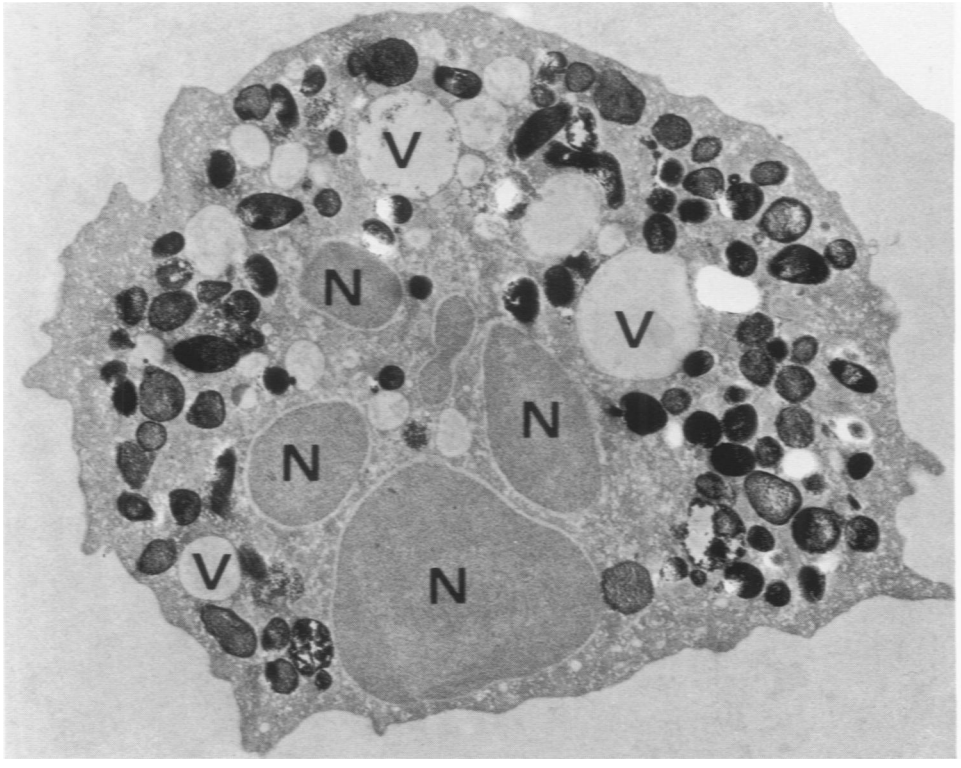

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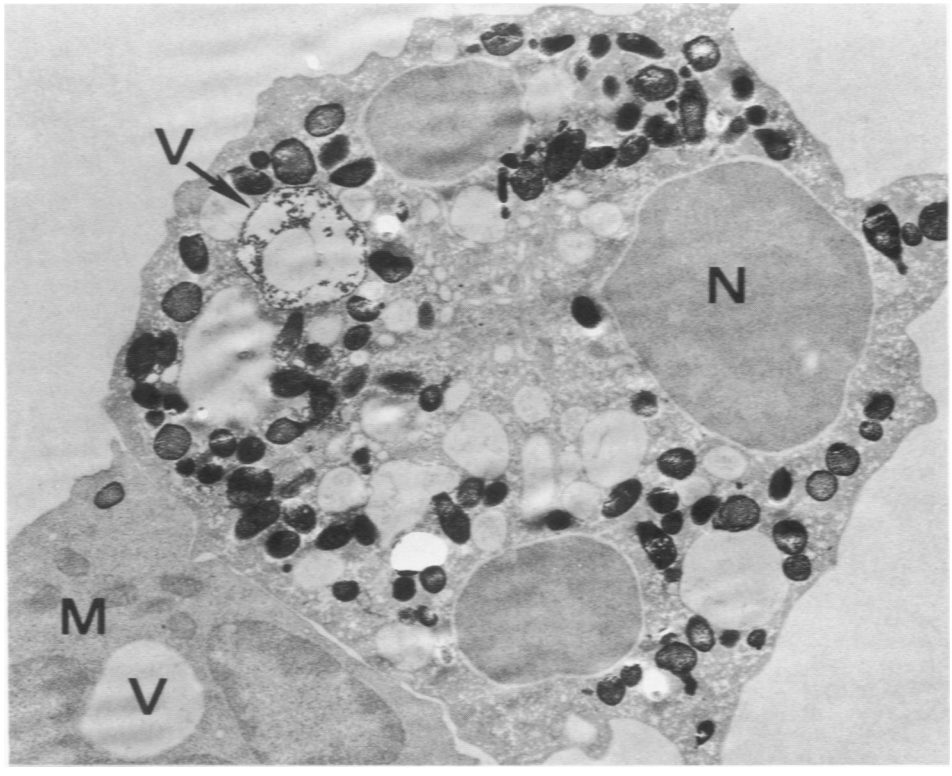
Fig 3—PMN from a sample of leukocytes exposed to PMA at a final concentration of 1 μg/ml for 30 minutes and prepared for study in the same manner as the cell in the previous illustration. One of the large vacuoles (*V*) generated by the action of PMA contains reaction product of myeloperoxidase activity. The rest of the vacuoles in the cytoplasm are devoid of reaction product which is limited primarily to intact azurophilic lysosomes. A monocyte (*M*) in this section contains a vacuole and one lysosome with myeloperoxidase reaction product. Monocytes appear as sensitive as PMN's to the action of PMA, but develop fewer vacuoles (× 14,200).

Fig 4—PMN from a control sample of normal leukocytes combined with thorium dioxide for 10 minutes. Particles of thorium dioxide are evident on the cell surface and in small vesicles (*Ve*) throughout the cytoplasm. Storage organelles in the cytoplasm appear unaltered (× 16,100).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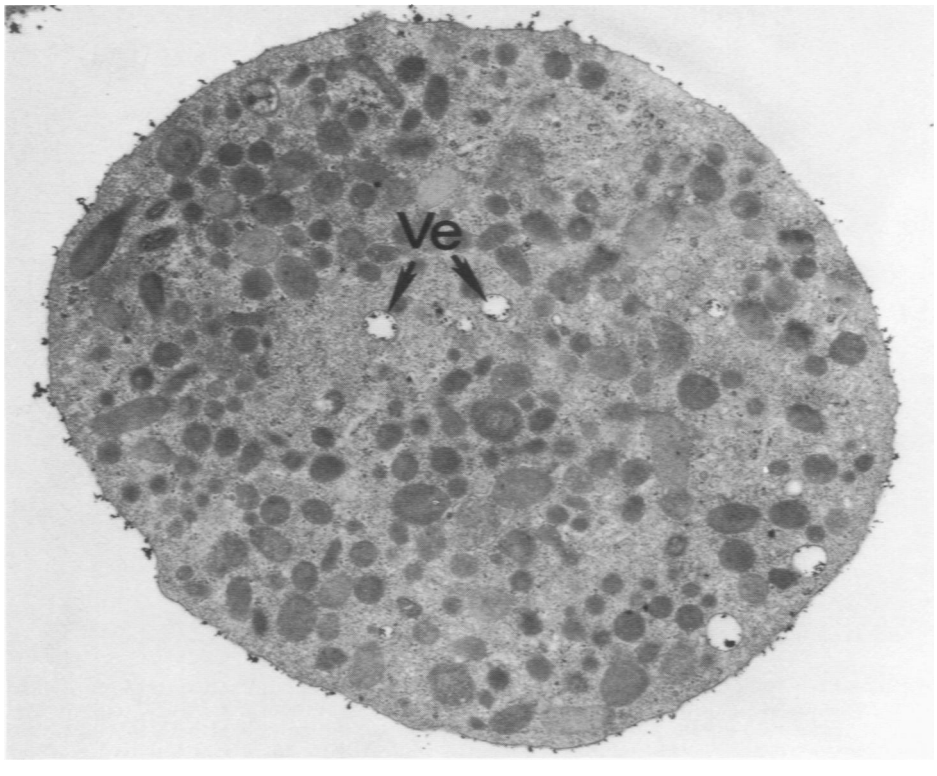
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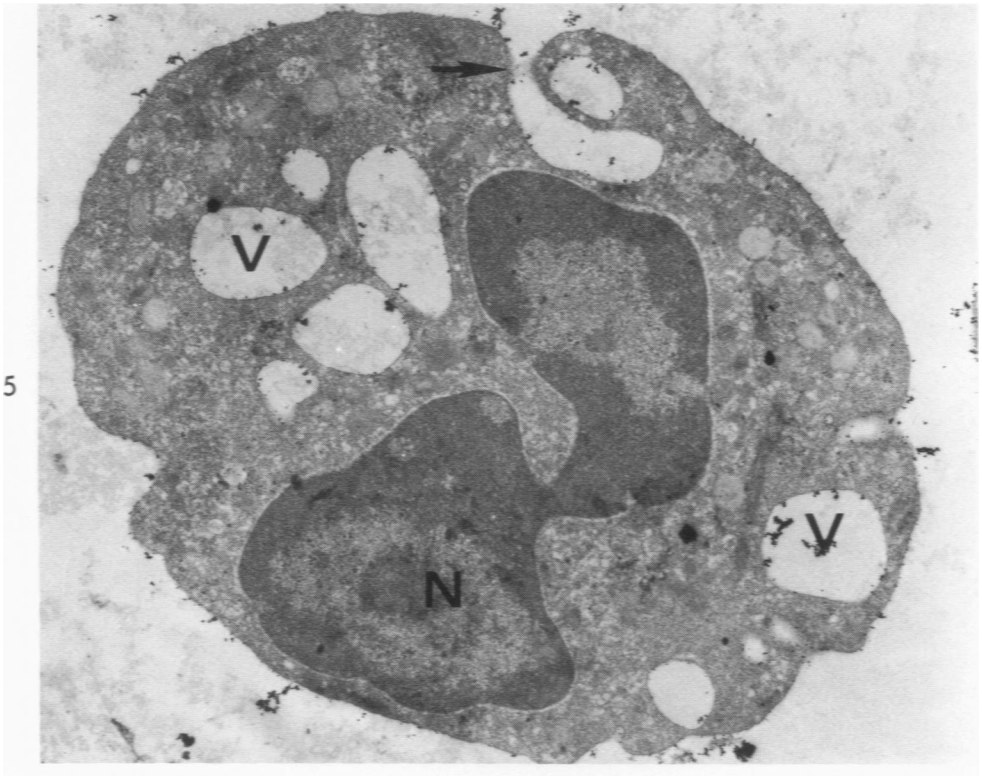

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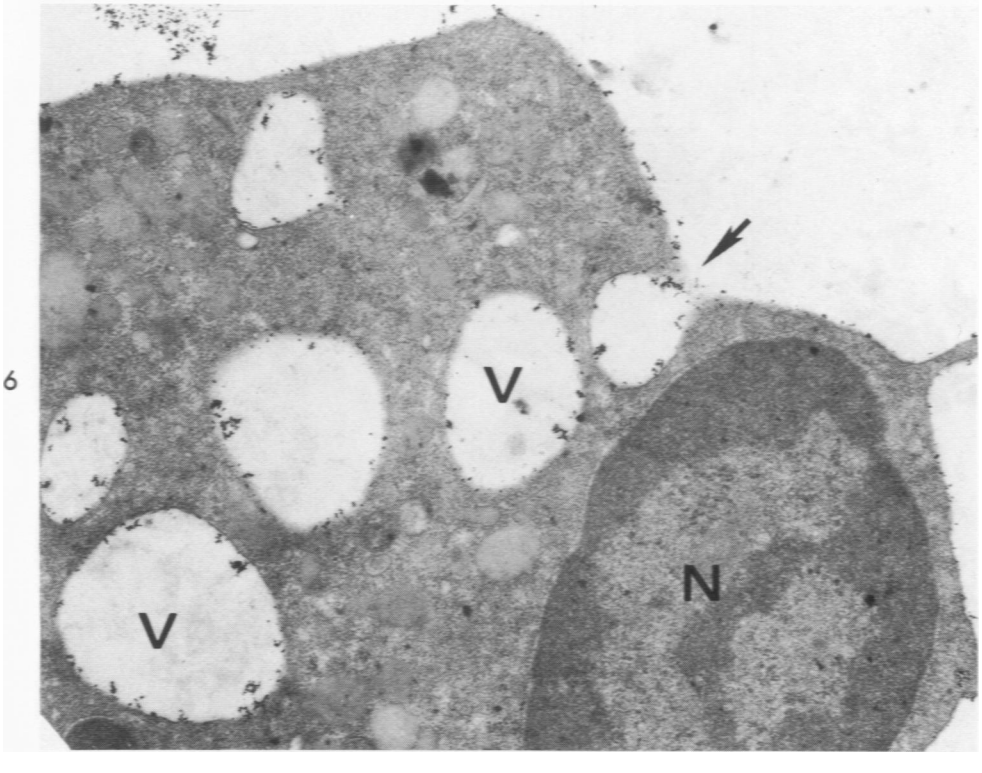
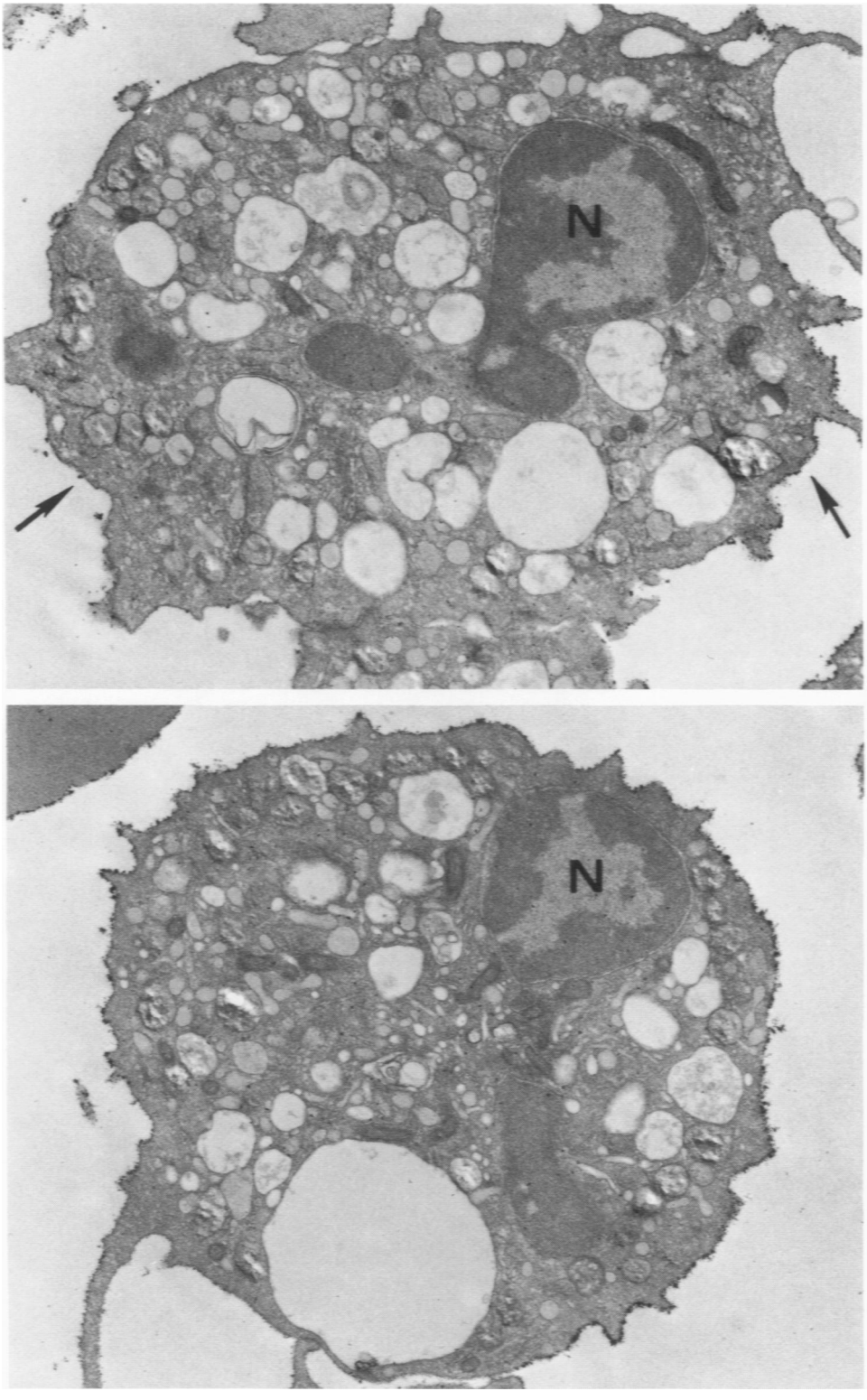
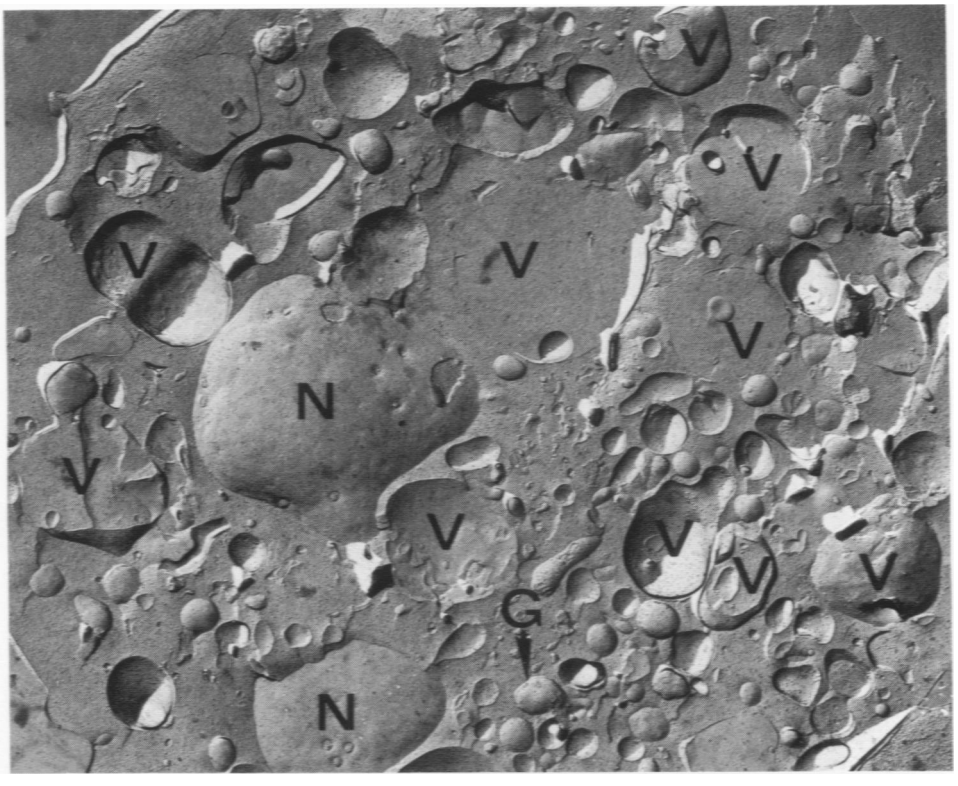


6

Figs 5 and 6—The PMN in **5** is from a sample of leukocytes combined with thorium dioxide for 5 minutes before incubation with PMA at a final concentration of 100 ng/ml for 10 minutes. Figure **6** reveals a PMN from a leukocyte sample combined with thorium dioxide at the same time PMA was added at a final concentration of 100 ng/ml and fixed after incubation for 10 minutes. Thorium dioxide particles are present on the surfaces of the cells and in all the cytoplasmic vacuoles. Areas of the cell surfaces apparently undergoing invagination and vacuole formation are evident in both examples (*arrow*) (**5,** × 16,000; **6,** × 41,500).



Figs 7 and 8—PMNs from samples of leukocytes exposed to 1 μg and 100 ng of PMA respectively and fixed after 10 minutes of incubation in solutions containing lanthanum nitrate. Particles of the electron dense stain coat the cell surfaces (*arrow*) but are not evident on the membranous linings of vacuoles present in the cytoplasm (**7**, × 15,000; **8**, × 13,500).



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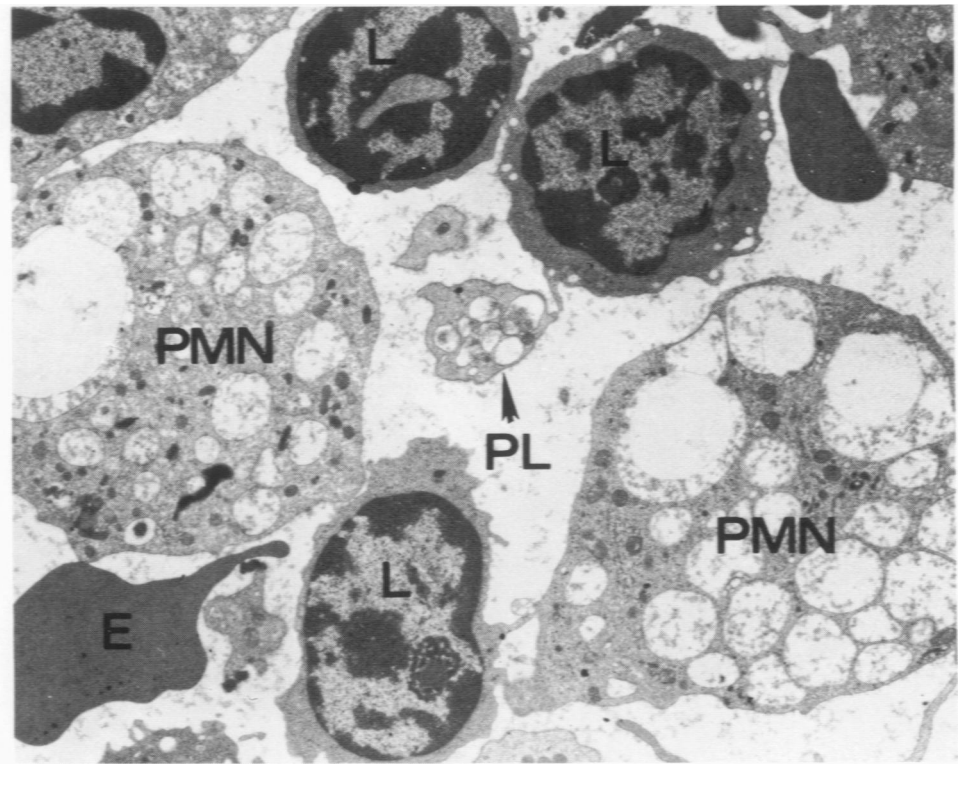

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Fig 9—Replica of a freeze-fractured PMA treated neutrophil. Large vacuoles (*V*) cleaved in various planes are dispersed throughout the cytoplasm. The membanes of the vacuoles, granules (*G*), nuclear lobes (*N*) and cell surface do not differ from the fracture faces of membranes observed in untreated PMNs (× 17,000). **Fig 10**—Random section through a leukocyte sample exposed to PMA at a concentration of 10 ng/ml and fixed after 1 hour of incubation. Lymphocytes (*L*) appear unaffected. Erythrocytes (*E*) may develop slight alterations in surface configuration, but are not damaged by the agent. PMNs and platelets (*Pl*) are very sensitive to the action of PMA, and develop multiple cytoplasmic vacuoles (× 6200).