LOCALIZATION OF D-AMINO ACID OXIDASE ON THE CELL SURFACE OF HUMAN POLYMORPHONUCLEAR LEUKOCYTES

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ABSTRACT

The ultrastructural localization of D-amino acid oxidase (DAO) was studied cytochemically by detecting sites of hydrogen peroxide production in human polymorphonuclear leukocytes (PMNs). Reaction product, which forms when cerous ions react with H_2O_2 to form an electron-dense precipitate, was demonstrated on the cell surface and within the phagosomes of phagocytically stimulated cells when D-amino acids were provided as substrate. Resting cells showed only slight activity. The competitive inhibitor D,L-2-hydroxybutyrate greatly reduced the D-amino acid-stimulated reaction while KCN did not. The cell surface reaction was abolished by nonpenetrating inhibitors of enzyme activity while that within the phagosome was not eliminated. Dense accumulations of reaction product were formed in cells which phagocytosed *Staphylococcus aureus* in the absence of exogenous substrate. No reaction product formed with *Proteus vulgaris* while an intermediate amount formed when *Escherichia coli* were phagocytosed. Variation in the amount of reaction product with the different bacteria correlated with the levels of D-amino acids in the bacterial cell walls which are available for the DAO of PMNs. An alternative approach utilizing ferricyanide as an electron acceptor was also used. This technique verified the results obtained with the cerium reaction, i.e., the DAO is located in the cell surface and is internalized during phagocytosis and is capable of H_2O_2 production within the phagosome. The present finding that DAO is localized on the cell surface further supports the concept that the plasma membrane is involved in peroxide formation in PMNs.

KEY WORDS D-amino acid oxidase · cell surface · polymorphonuclear · leukocytes · phagocytosis

Phagocytosis by polymorphonuclear leukocytes (PMNs)[1] is accompanied by changes in cellular metabolism which include increases in oxygen consumption, hexose monophosphate shunt activity, and production of hydrogen peroxide (24, 16, 18). Recently, Briggs et al. (3) demonstrated cytochemically that PMNs have a surface NADH oxidase which is stimulated by phagocytosis or other perturbations of the plasma membrane. The NADH oxidase internalization may be an important event since this enzyme can generate H_2O_2 within the phagosome for utilization by myeloperoxidase for bactericidal activity (17).

Human PMNs have other enzymes which are

[1] *Abbreviations used in this paper*: ATZ, 3-amino-1,2,4-triazole; DAB, 3-3′ diaminobenzidine; DAO, D-amino acid oxidase; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; PCMBS, parachloromercuribenzenesulfonate; PMNs, polymorphonuclear leukocytes; and PS, polystyrene beads.

capable of H_2O_2 generation. Cline and Lehrer (6) reported on the presence of a D-amino acid oxidase (DAO) EC 1.4.3.3. They suggested that since certain bacteria contain D-amino acids in nonpolypeptide linkage in their cell walls, these compounds may serve as a natural substrate for generation of H_2O_2 and subsequent bactericidal activity by PMNs. The presence of DAO in PMNs was verified by Eckstein et al. (11); however, they found no difference in the activity of the enzyme in normal subjects and patients with chronic granulomatous disease. In addition, D-alanine and D-threonine failed to elicit an increase in hexose monophosphate shunt activity in resting PMNs or those phagocytosing polystyrene particles (7). On the basis of the two latter reports, it has been suggested that DAO does not play a major role in bactericidal activity in human PMNs (11, 7).

While DAO may not be the major source of H_2O_2 within PMNs, the importance of the enzyme in the killing of certain bacteria (those with high levels of available cell wall D-amino acids) has not been determined. The subcellular localization of DAO has not been unequivocally demonstrated (6, 11).

The advent of a cytochemical procedure for detecting sites of H_2O_2 production (3) permits a new approach to the study of DAO localization within PMNs. Briefly, when cerium ions are included in the cytochemical medium, a precipitate of cerium perhydroxide forms at the sites of H_2O_2 generation. Localization of DAO was also studied with a different approach; this involved use of ferricyanide as a terminal electron acceptor to produce an insoluble copper ferrocyanide complex at the site of reduction (for a review of oxidoreductase cytochemistry see Hanker, 12). Both techniques give the same localization of DAO, that is, the external face of the plasma membrane and the internal face of the phagosome membrane.

MATERIALS AND METHODS

Isolation of Cells

Human PMNs were isolated by a modification of the technique of Harris (14). Clean glass cover slips (22 mm diameter, no. 2 thickness) were flooded with blood from a pricked finger, placed upright on plastic bars and incubated in a moist chamber for 5 min at room temperature followed by 30–40 min at 37°C. The clot was removed, and erythrocytes were rinsed from the cover slips by repeated dipping in Hanks' balanced salt solution (HBSS), pH 7.4 at 4°C. These preparations routinely gave >90% PMNs, the remainder being monocytes and eosinophils.

Phagocytosis

Cells on cover slips were allowed to phagocytize polystyrene beads (PS, 1.1 μm diameter) (Dow Chemical Co., Midland, Mich.). The beads were prepared by extensive dialysis against phosphate-buffered saline (PBS) for 72 h. Cover slip preparations of PMNs were overlaid with 0.5 ml of the PS suspension (1:20 dilution of a 2.5% dialyzed stock prepared in HBSS with 1 mg/ml of additional glucose). This gives an excess of beads to cells.

In some experiments, bacteria were used for phagocytosis. Bacteria were selected on the basis of the levels of available D-amino acids in their cell walls. Enzymatic analysis has shown that *Staphylococcus aureus->Escherichia coli>Proteus vulgaris* with respect to their available D-amino acid content (6). Bacteria were inoculated with a wire loop into 10 ml of a mixture of equal parts of brain heart infusion, Sabouraud dextrose broth, and tryptose phosphate broth (Difco Laboratories, Detroit, Mich.). Bacteria were concentrated by centrifugation, washed in HBSS, and resuspended in fetal calf serum (FCS). The bacteria were opsonized with a 15-min incubation of FCS at 37°C, in order to render them more susceptible to phagocytosis, and resuspended in HBSS with 50 mM 3-amino-1,2,4-triazole (ATZ) (Aldrich Chemical Co., Inc., Milwaukee, Wis.). Use of ATZ in this step as well as in the subsequent cytochemical reaction was to inhibit any endogenous catalase activity which could remove H_2O_2 as quickly as it formed. Cover slip preparations of PMNs were overlaid with 0.5 ml of the bacterial suspension. The concentration of bacteria was not quantitated, but the washed pellets were resuspended in enough HBSS with ATZ to yield a visually turbid suspension. In each case, however, there were excess bacteria to PMNs.

Cover slips receiving either PS or bacteria were incubated for 20 min at 37°C in a moist chamber. After incubation, excess particles were removed by repeated rinsing in HBSS at 4°C. Resting PMNs were incubated in HBSS plus glucose (1 mg/ml) or HBSS with ATZ (50 mM) for 20 min at 37°C and then rinsed in the same way as the phagocytosing cells.

Cerium Reaction

Unfixed cells, phagocytosing or resting, were briefly washed in Tris-maleate buffer (0.1 M, pH 7.5) with 5% sucrose and then preincubated for 10 min at 37°C in Tris-maleate, pH 7.5, with 5% sucrose containing 1 mM ATZ. Unfixed cells were used in H_2O_2 localization experiments for the following reasons: (*a*) preliminary studies showed that even brief fixation with glutaraldehyde or freshly depolymerized paraformaldehyde diminished the amount of reaction product formed, (*b*) good morphological detail was preserved without fixation before the cytochemical reaction. The final cytochemical medium containing 0.1 M Tris-maleate, pH 7.5, with 5% sucrose, 10 mM ATZ, 1 mM $CeCL_3$, and 1 mM D-amino acid (D-alanine, D-valine, or D-phenylalanine)

(Sigma Chemical Co., St. Louis, Mo.). Cover slip preparations were incubated for 20–30 min at 37°C. The cytochemical incubation media were prepared just before use and filtered (0.45 μm, Millipore Corp., Bedford, Mass.) in order to remove any cerium hydroxide which may have formed at pH 7.5. After incubation, coverslips were rinsed in 0.1 M Tris-maleate buffer, pH 7.5 with 5% sucrose. Cells were fixed in 2% glutaraldehyde-0.1 M cacodylate buffer, pH 7.3, with 5% sucrose for 60 min at 4°C, followed by a wash in 0.1 M cacodylate buffer, pH 6.0, with 5% sucrose. This acidic wash removes any cerium hydroxide precipitate which may form during incubation (3). Cells were subsequently washed 1 h to overnight in 0.1 M cacodylate, pH 7.3 with 5% sucrose and postfixed in 2% OsO_4–0.1 M cacodylate for 60 min at room temperature.

Ferricyanide Reduction

Cover slip preparations of resting or phagocytosing cells were fixed for 10 min in freshly prepared 2% formaldehyde (depolymerized from paraformaldehyde) in 0.1 M cacodylate buffer, pH 7.3, containing 5% sucrose at 4°C. Cells were then washed 30–45 min in 0.1 M cacodylate with sucrose at 4°C followed by a brief wash in 0.1 M phosphate buffer, pH 7.3 with 5% sucrose. The cytochemical medium which consisted of 0.1 M phosphate buffer, pH 7.2, 2 mM potassium sodium tartrate, 3 mM copper sulfate, 0.5 mM potassium ferricyanide, 1 mM D-alanine, and 10% dimethylsulfoxide with 5% sucrose added was prepared immediately before use and adjusted to pH 7.2. The ferricyanide medium for localization of DAO was a modification of existing techniques for localization of other oxidoreductases (12). After a 30-min incubation at 37°C, the cover slips were rinsed in cold phosphate buffer followed by 0.05 M acetate buffer, pH 5.6. The cover slips were then treated with 3-3′-diaminobenzidine (DAB) in acetate buffer (0.5 mg/ml) for 30 min at room temperature according to the procedure of Hanker et al. (13) for osmiophilic polymer generation (DAB amplification). The cover slips were rinsed in cacodylate-sucrose and treated with 2% OsO_4–0.1 M cacodylate for 60 min at room temperature. Alternatively, cells were fixed in 2% glutaraldehyde-0.1 M cacodylate, pH 7.3, with 5% sucrose for 30 min at 4°C and then washed in several changes of 0.1 M cacodylate for 30 min at 4°C before DAB treatment.

Electron Microscopy

After osmication, cells were dehydrated in ethanol and embedded in Epon 812. Capsules containing Epon were inverted over the cover slips and polymerized for 24 h at 60°C. Blocks containing cells were separated from the cover slips by immersing blocks, taken directly from the oven, into liquid nitrogen. Thin sections were cut with a diamond knife on an LKB Ultratome (LKB Instruments, Inc., Rockville, Md.) and examined either unstained or stained with aqueous uranyl acetate and lead citrate in a Philips 200 electron microscope operated at 60 kV.

Controls

The various controls employed will be discussed at appropriate points in the Results section.

RESULTS

H_2O_2 Localization

The localization of DAO, based on detection of sites of H_2O_2 production, was determined in mature human PMNs. When unfixed living PMNs were allowed to phagocytose PS and were subsequently treated in the complete cerium cytochemical medium, a characteristic pattern of reaction product was observed. Reaction product was found on the cell surface (external face of the plasma membrane) and on the internal surface of the phagosome membrane (Fig. 1). The amount of reaction product at the cell surface was somewhat variable, i.e., in some cells it completely covered the surface, while in others it was discontinuous or localized in a single region of the membrane. Reaction product was also seen in channels connecting incompletely closed phagocytic vacuoles with the surface. Vesicles arising from surface infoldings or portions of channels also showed reaction product in thin section. Phagosomes which were negative were occasionally observed. Cerium precipitates were restricted to the plasma membrane or phagosome and were not observed in primary or secondary granules or other cytoplasmic organelles. Routinely, D-alanine was used as substrate, but comparable results were obtained with D-phenylanine. D-valine, on the other hand, was not an effective substrate under the conditions employed in these experiments.

Resting cells (live unfixed PMNs not exposed to PS) showed only occasional deposits of reaction product after incubation in the complete cytochemical medium (Fig. 2). As in the positive experiments, no cytoplasmic organelles showed reaction product. Only occasional deposits were observed in surface-connected channels or vesicles.

In any given positive experiment, there may be a range in the amount of reaction product. Also, many cells lack reaction product altogether. Direct counts reveal that approx. 40% of the PMNs show reaction with D-alanine as substrate.

Substrate Dependence

When D-amino acids were omitted from the

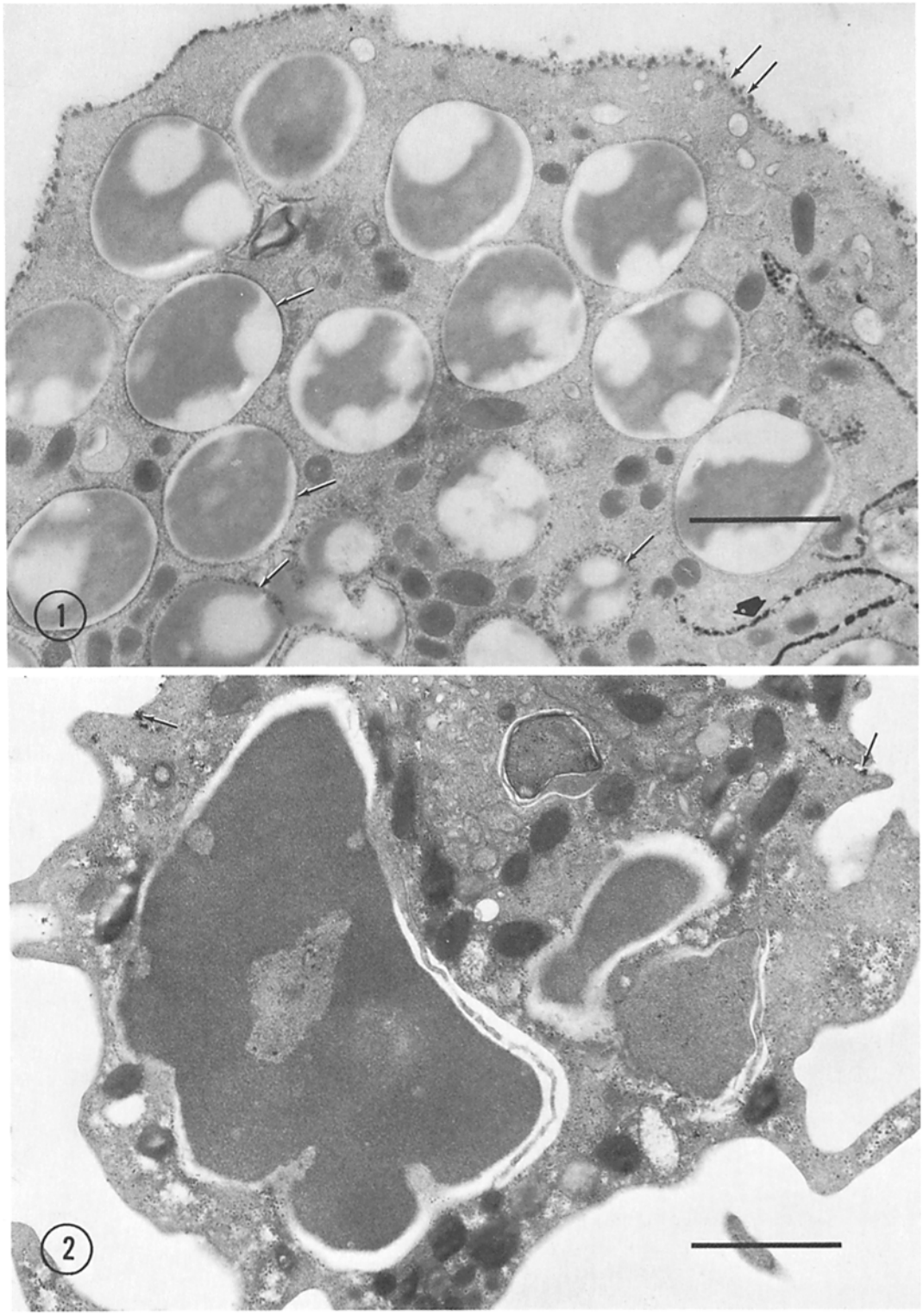


FIGURE 1 A human polymorphonuclear leukocyte (PMN) which was stimulated by phagocytosing PS before hydrogen peroxide localization. The cell was incubated in the complete cytochemical medium containing Ce^{+++}, ATZ, and Tris-maleate buffer with D-alanine as substrate. Note reaction product found on the cell surface (double arrows) and on the inner face of the phagosome membrane (single arrows). Reaction product is also found in channels derived from invaginations of the plasma membrane (bold arrows). × 23,000. Bar, 1 μm.

FIGURE 2 A PMN incubated in the complete cytochemical medium with D-alanine as substrate but without phagocytic stimulation. Note that only an occasional deposit of reaction product is seen on the cell surface (arrows). × 23,000. Bar, 1 μm.

cytochemical medium, the amount of reaction product was dramatically reduced in phagocytosing cells (Fig. 3). In addition to D-amino acids, L-alanine was used as a substrate. Use of this substrate results in a localization pattern similar to that seen with D-amino acids. The localization of DAO was also similar to that reported for NADH oxidase (3). In the present study, the localization of NADH oxidase was determined in PS-stimulated PMNs by the method of Briggs et al. (3). Our results verify the earlier observation that the reaction product is localizable to the external face of the plasma membrane and the internal surface of the phagosome membrane. In addition, the elimination of NADH from the medium dramatically reduces the amount of reaction product in PS-stimulated PMNs. Although the virtual absence of reaction product in the no substrate controls, and the substrate-dependency of the reactions, imply that detection of DAO might be separable from detection of NADH oxidase and L-amino acid oxidase, these observations do not, however, exclude the possibility that the same enzyme system is involved in the oxidation of each substrate.

Cyanide Insensitivity

Phagocytosis and the accompanying increase in O_2 consumption and H_2O_2 production are insensitive to 1 mM KCN (16). In addition, Cline and Lehrer (6) have shown that the same concentration of KCN has no inhibitory effect on D-alanine oxidation by PMNs. The effect of cyanide on the cytochemical detection of D-alanine stimulated H_2O_2 production in PMNs was determined by including 1 mM KCN in all the incubation media. The results show that 1 mM KCN had no detectable inhibitory effect on the cytochemical reaction in PMNs allowed to phagocytose PS (Fig. 4).

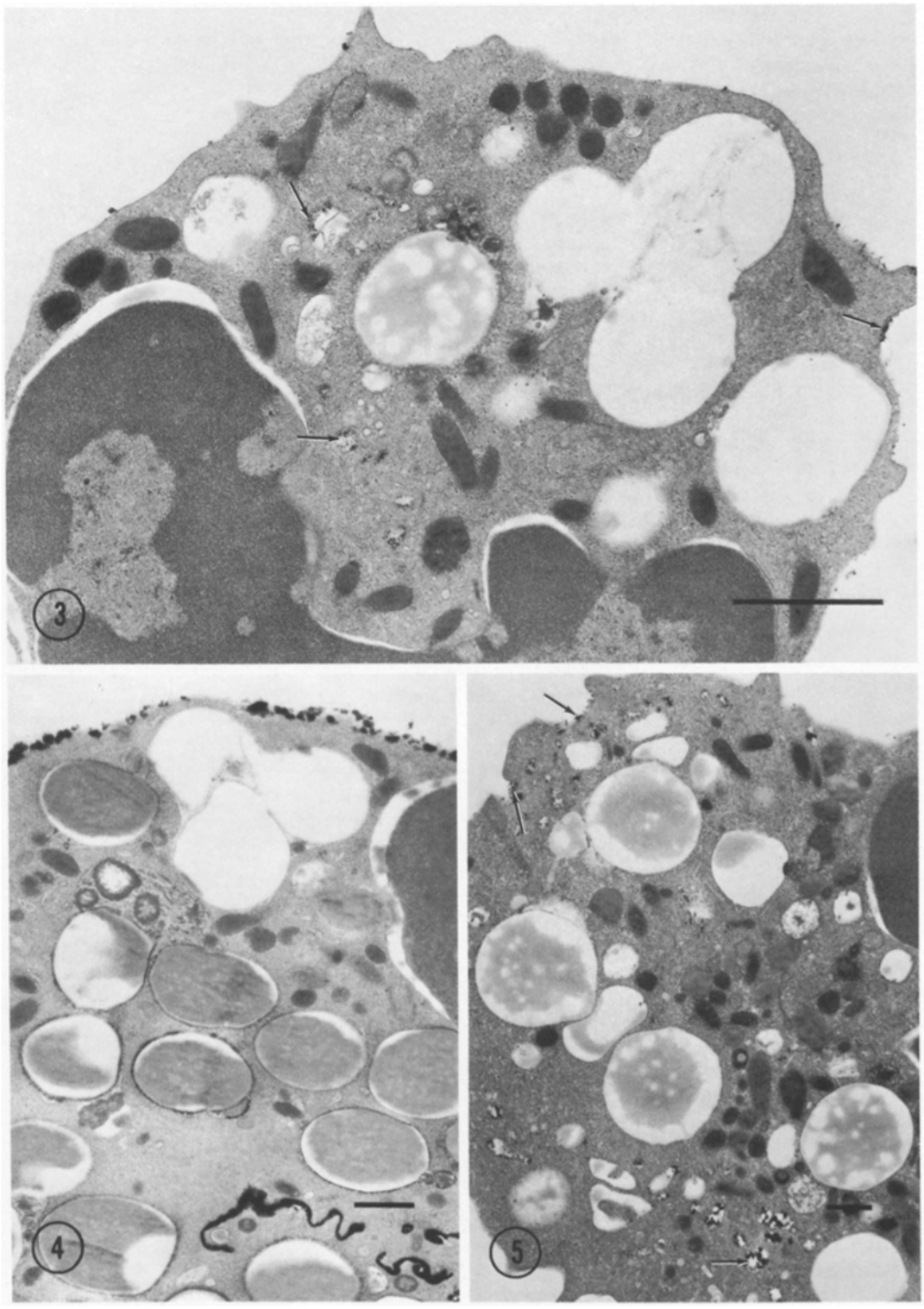
Effect of Inhibitors

Enzyme inhibitors were used to study the specificity of the reaction and to show that cytochemically detectable H_2O_2 was generated enzymatically. Cline and Lehrer (6) have shown that 0.01 M D,L-2-hydroxybutyrate inhibits 70% of the D-alanine-stimulated uptake of O_2 by PMN homogenates. Furthermore, Dixon and Kleppe (10) have shown 0.01 M D,L-2-hydroxybutyrate to be a highly effective competitive inhibitor of purified pig kidney DAO. Therefore, 0.01 M D,L-2-hydroxybutyrate (Sigma Chemical Co.) was included in all the incubation media. The results show that inclusion of this inhibitor in the cytochemical media virtually eliminates the presence of cerium precipitates in PS-phagocytosing PMNs (Fig. 5).

Two nonspecific, nonpenetrating inhibitors were used to confirm the localization of DAO on the cell surface. As in the study by Briggs et al. (3), it was reasoned that if nonpenetrating inhibitors could block reaction product formation on the surface of the cell, then the enzyme is probably located on the plasma membrane. Parachloromercuribenzenesulfonate (PCMBS) (Sigma Chemical Co.), a nonpenetrating sulfhydryl reagent which has been shown to inhibit glucose transport in erythrocytes (25), was selected. PMNs were allowed to phagocytose PS; then unfixed preparations were preincubated in 10 mM PCMBS in 0.1 M Tris-maleate buffer, pH 7.5, with 5% sucrose for 5 min at 37°C before incubation in the normal cytochemical medium containing 10 mM PCMBS. Essentially no reaction product was detected on the surface; however, some phagosomes were positive. The presence of nonreactive phagosomes may be accounted for by incompletely closed phagosomes which were continuous with the surface, thus allowing the inhibitor to block the reaction. The second nonpenetrating enzyme inhibitor was the diazonium salt of sulfanilic acid which has been shown to affect other surface and ectoenzymes of PMNs (8, 9). The inhibitor was prepared by the procedure of Berg (2); unfixed cells were allowed to phagocytose PS and then were preincubated in 3.5 mM inhibitor in phosphate-buffered saline (PBS) for 10 min at 37°C. Subsequently, cells were washed several times in PBS, then in Tris buffer, before cytochemical incubation with D-alanine as substrate. The results were similar to those obtained with PCMBS as inhibitor, i.e., accumulation of surface reaction product was eliminated while many phagosomes had reaction product (Fig. 6). The use of inhibitors supports the contention that D-amino acid-stimulated accumulation of reaction product was enzymatically mediated. Furthermore, use of the nonpenetrating inhibitors provides evidence that DAO has a cell surface localization in human PMNs.

Phagocytosis of Bacteria

Bacteria containing high concentrations of available cell wall D-amino acids have been shown to serve as substrate for PMN homogenates in O_2 consumption studies (6). Experiments were con-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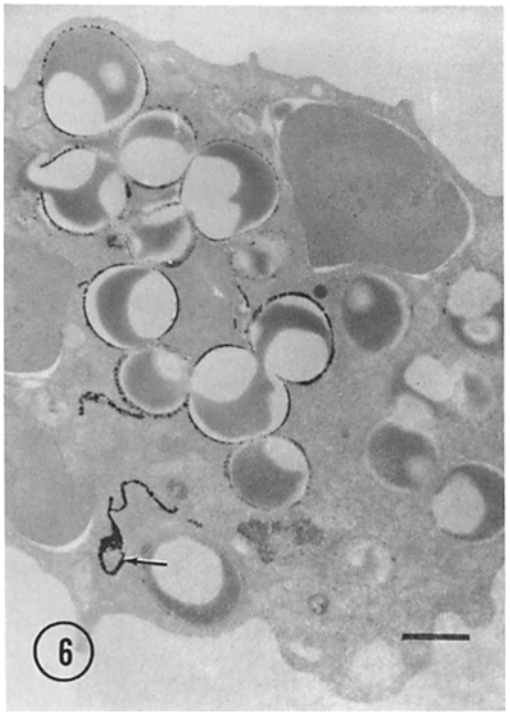


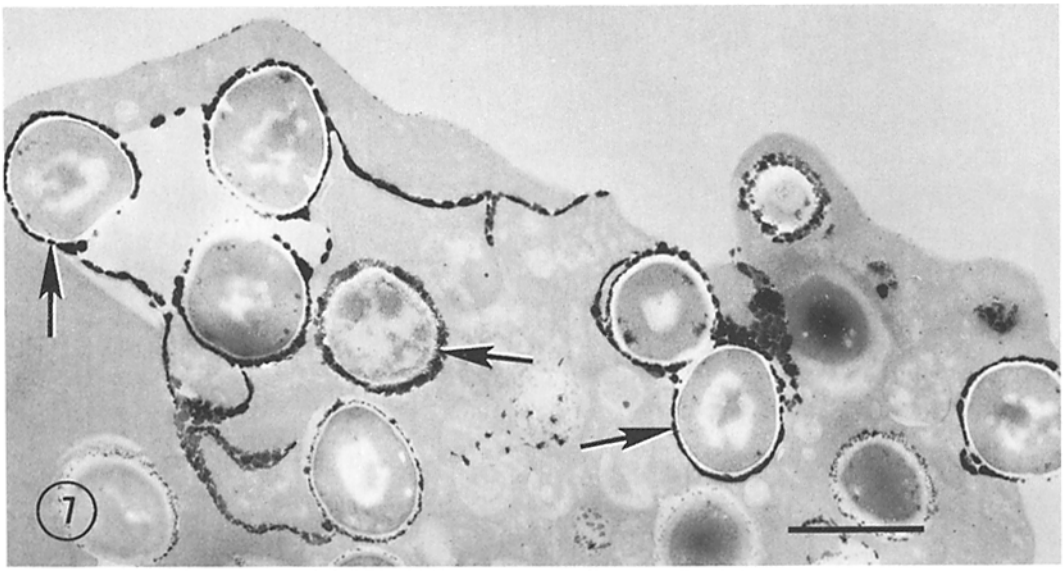
FIGURE 6 A PMN incubated with the diazotized salt of sulfanilic acid (3.5 mM) after phagocytosis but before incubation in the complete cytochemical medium with D-alanine as substrate. This nonpenetrating inhibitor eliminates the cell surface reaction without affecting the reaction in phagocytic vacuoles or some channels (arrow). No counterstain. × 9,100. Bar, 1 μm.

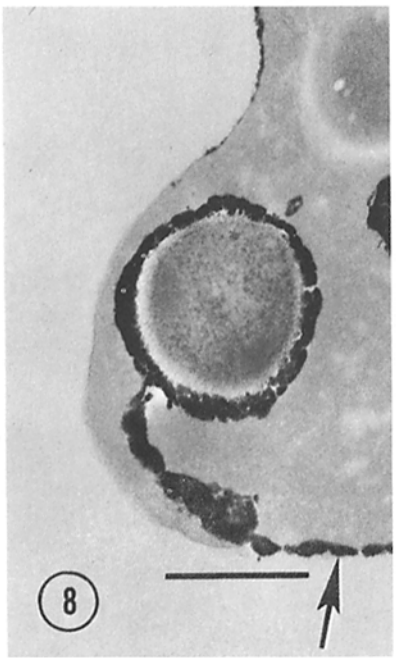
ducted to see if similar results could be obtained in the cytochemical assay for H_2O_2 detection described in the present study. A positive reaction was observed in PMNs which phagocytosed *S. aureus* and were subsequently incubated unfixed in the cytochemical medium from which substrate was omitted. Heavy deposits of cerium precipitate were detected within phagosomes containing these bacteria. Reaction product was restricted to the phagosome membrane and did not necessarily surround the entire bacterium (Fig. 7). Additionally, reaction product could be found on the surface of cells phagocytosing *S. aureus*. Surface reaction is thought to arise in two ways: (*a*) from incompletely closed phagosomes in which substrate derived from the cell wall diffused to the surface via channels (Fig. 8) or (*b*) from a reaction initiated on the plasma membrane by bacteria which adhere to the surface but are not internalized during the course of the incubation (Fig. 9). Lesser amounts of reaction product were observed when *E. coli* were phagocytosed and cells were subsequently reacted in medium lacking substrate. Reaction product, even though less abundant, was observed to be on the internal face of the phagosome membrane (Fig. 10). Only occasional surface deposits were detected in cells which had phagocytosed *E. coli*. Essentially no reaction product was observed when *P. vulgaris* were phagocytosed and subsequently reacted unfixed in the cytochemical medium lacking substrate (Fig. 11). These results are in agreement with the biochemical study of Cline and Lehrer (6). In their study, *S. aureus* could serve as substrate for DAO of human PMNs, while with purified hog kidney DAO bacteria served as substrate in the following order of reactivity: *S. aureus* > *E. coli* > *P. vulgaris*. Furthermore, *P. vulgaris* was only slightly active as a substrate for this purified enzyme. It seems reasonable to attribute the levels of reaction product observed in this study to the amounts of available D-amino acids in the cell walls of the various bacteria. These observations further support our contention that human PMNs

FIGURE 3 A PMN which was phagocytically stimulated with PS and incubated in a control medium lacking D-amino acid. Only an occasional deposit of reaction product is seen on the cell surface or internal vesicles (arrows). Note that the PS within the phagosomes may be completely extracted or only partially extracted during processing for electron microscopy. × 23,000. Bar, 1 μm.

FIGURE 4 A phagocytically stimulated PMN incubated in the complete cytochemical medium with D-alanine as substrate and with 1 mM KCN included. This level of KCN was not inhibitory to the reaction. × 17,000. Bar, 0.5 μm.

FIGURE 5 Incubation of phagocytically stimulated cells in the complete medium with D-alanine as substrate but with the competitive inhibitor 10 mM D,L-2-hydroxybutyrate added results in almost complete elimination of reaction product. Only small scattered sites of reaction product are evident (arrows). × 15,200. Bar, 0.5 μm.





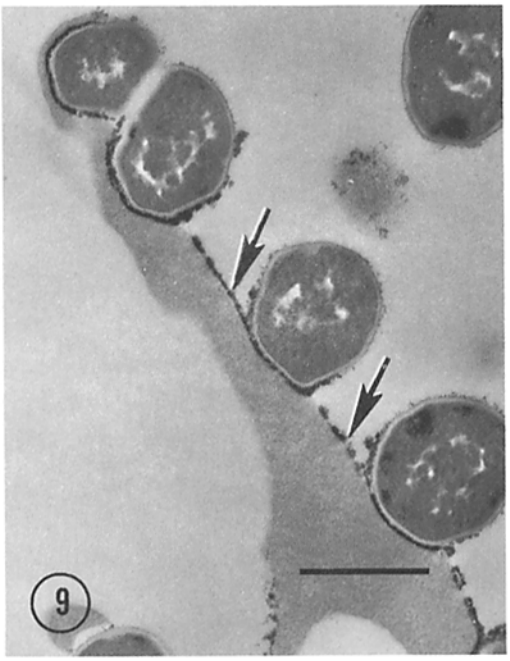


FIGURE 7 Portion of a PMN which phagocytosed *S. aureus* before incubation in a cytochemical medium containing Ce^{+++}, ATZ, and Tris-maleate buffer but lacking exogenous D-amino acids. Note production of hydrogen peroxide, as detected by formation of reaction product, within the phagocytic vacuoles (arrows). No counterstain. × 18,200. Bar, 1 μm.

FIGURE 8 Portion of a PMN which phagocytosed *S. aureus* and then was reacted as in Fig. 7. Note area with reaction product on the cell surface (arrow). This type of surface reaction may arise from the phagosome via channels connecting phagosome and surface. No counterstain. × 36,800. Bar, 0.5 μm.

FIGURE 9 Portion of a PMN incubated with *S. aureus* and then reacted as in Fig. 7. Note area with reaction product on the cell surface (arrows). This type of surface reaction may develop when the bacteria adhere to the cell surface but are not internalized during the course of the incubations. While slight Ce^{+++} deposits may be found on the free surface of the adherent bacteria, the dense accumulations of reaction product are on the PMN cell surface. No counterstain. × 34,000. Bar, 0.5 μm.

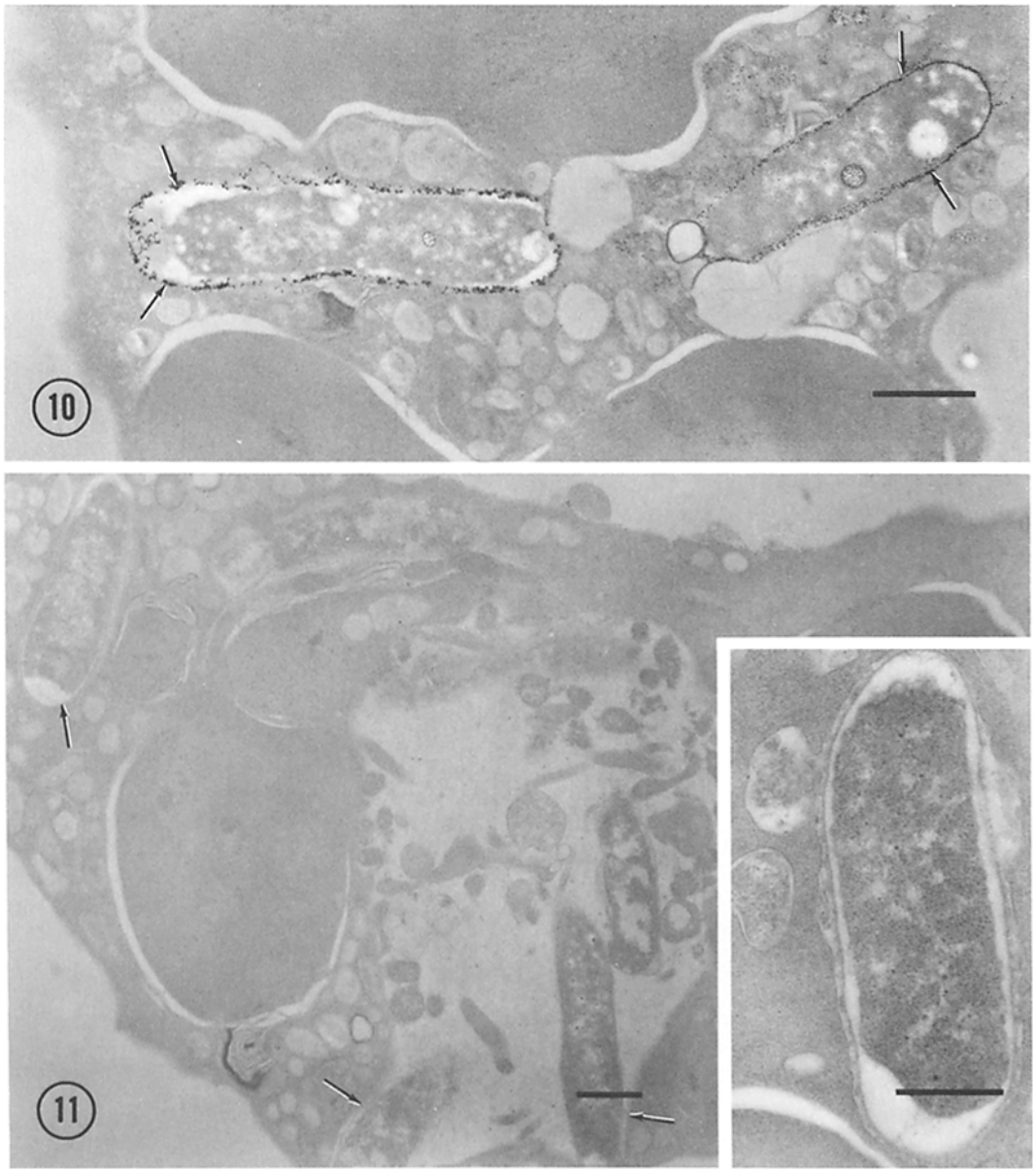


FIGURE 10 A PMN which has phagocytosed *E. coli* before incubating in a substrate-free medium as in Fig. 7. Reaction product is found on the internal face of the phagosome membrane (arrows). Note that reaction product is qualitatively less than that found when *S. aureus* were phagocytosed and reacted in the same manner. For comparison, see Figs. 7, 8, and 9. No counterstain. × 14,000. Bar, 1 μm.

FIGURE 11 A PMN which has phagocytosed *P. vulgaris* before incubating in a substrate-free medium as in Fig. 7. Note that phagosomes lack reaction product under these conditions (arrows). No counterstain. × 9,600. Bar, 1 μm. *Inset*: Higher mag. of *P. vulgaris* within phagosome. × 28,800. Bar, 0.5 μm.

possess a cell surface DAO which can be internalized during phagocytosis.

Ferricyanide Reduction

Cytochemical demonstration of several oxidoreductases has been achieved by various methods which rely on ferricyanide reduction and precipitation by copper to yield an electron-dense copper ferrocyanide reaction product (for review, see Hanker, reference 12). A similar approach was

undertaken in this study to develop a technique for DAO localization to augment the H_2O_2 localization procedures. Preliminary studies showed that when unfixed cells were reacted with the ferricyanide medium, very poor cellular preservation was achieved, and often the cells were almost completely destroyed. Subsequently, a 10-min fixation in 2% formaldehyde at 4°C was employed. This procedure aided in cellular preservation; however, the ultrastructure obtained was still inferior to that found when the cerium containing cytochemical medium was used. Furthermore, prefixation diminishes the number of reactive cells. This inhibitory effect of aldehyde fixation was confirmed with the cerium technique. These drawbacks notwithstanding, the results obtained with the ferricyanide cytochemical procedure support the results obtained in the H_2O_2 localization experiments. Prefixed cells incubated in the ferricyanide medium with D-alanine as substrate show reaction on the surface of the plasma membrane (Fig. 12). A surface reaction was observed with this procedure even when cells were not stimulated by phagocytosis; however, no reaction was observed in the absence of substrate (Fig. 13). In cells which phagocytosed PS before prefixation in formaldehyde, reaction product was observed on the inner face of the phagosome membrane (Fig. 14). Results with the ferricyanide procedure support those obtained with the H_2O_2 localization technique.

DISCUSSION

It is well known that phagocytically stimulated PMNs display metabolic alterations, for example, increases in oxygen uptake and hydrogen peroxide production. Two different mechanisms have received major attention as the explanation for these metabolic changes: activation of the enzymes NADH oxidase (1, 5, 16) and NADPH oxidase (19, 23). It has recently been shown by Briggs et

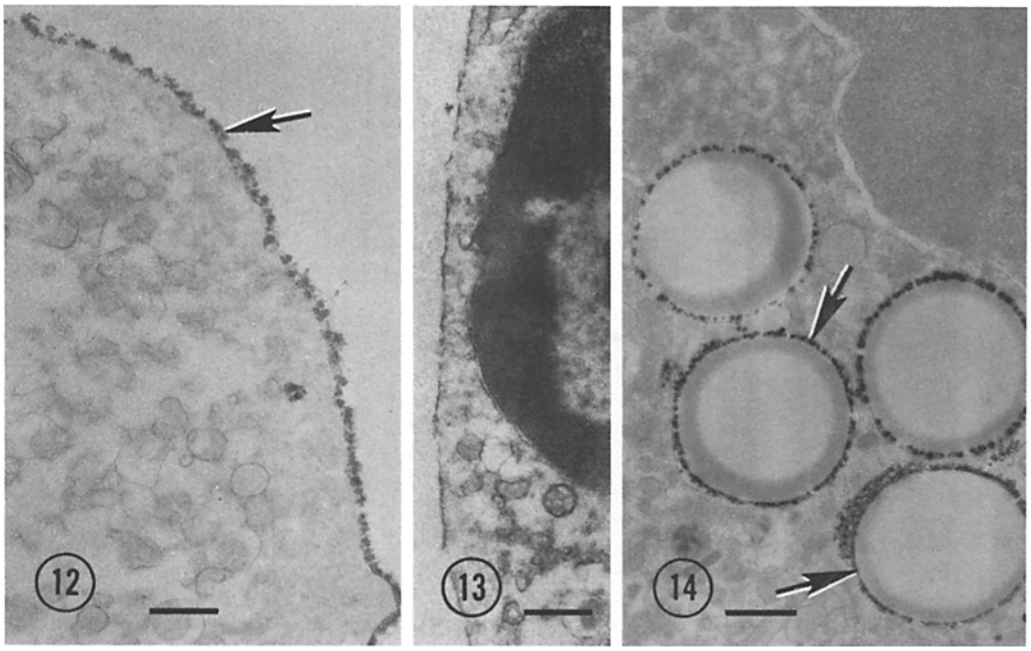


FIGURE 12 Resting cell showing sites of ferricyanide reduction on the cell surface (arrow). Incubated in the copper ferricyanide medium with D-alanine as substrate. Note that poor cellular preservation is achieved with this procedure. No counterstain. × 17,600. Bar, 0.5 μm.

FIGURE 13 Control resting cell incubated in the ferricyanide medium lacking substrate. Note the absence of reaction product. No counterstain. × 18,000. Bar, 0.5 μm.

FIGURE 14 Portion of a phagocytically stimulated PMN incubated in the copper ferricyanide medium with D-alanine as substrate. Reaction product is found on the internal face of the phagosome membrane (arrows). No counterstain. × 19,000. Bar, 0.5 μm.

al. (3) that NADH oxidase can be detected on the surface of PMNs. A surface localization and subsequent internalization of the enzyme during phagocytosis would lead to the generation of H_2O_2 within the phagosome. The relevance of hydrogen peroxide generation within the phagosome for reaction with myeloperoxidase and halide after degranulation has been recognized (22).

D-amino acid oxidase, which is present in PMNs, can also generate hydrogen peroxide (6); however, this enzyme has received little attention. The quantitative importance of DAO has been questioned since no difference was found in the activity of the enzyme from patients with chronic granulomatous disease and normal subjects (11); also, D-alanine and D-threonine failed to elicit an increase in hexose monophosphate shunt activity in phagocytosing PMNs when compared to resting cells (7). The subcellular localization of DAO has also been disputed; Cline and Lehrer (6) report that the enzyme is found in the granule fraction, while Eckstein et al. (11) report that it is in the soluble fraction. The reason for the discrepancy in these results is not clear; however, it should be pointed out that Cline and Lehrer did not present EM evidence that their granule preparation was without plasma membrane contamination. Also Eckstein et al. found the highest specific activity for DAO to be in a microsome fraction even though most of the activity was in a soluble fraction.

This paper focuses on a reinvestigation of the localization of DAO with a newly developed cytochemical technique for detecting the presence of H_2O_2. The results reported herein indicate that DAO is localized on the plasma membrane of human PMNs and that the enzyme can be internalized during phagocytosis. This interpretation is based on the following cytochemical results: (*a*) formation of reaction product in both cerium and ferricyanide techniques is substrate dependent; (*b*) cerium reaction product formation on the cell surface is blocked by the competitive inhibitor D,L-2-hydroxybutyrate and the less specific, nonpenetrating sulfhydryl enzyme inhibitors; and (*c*) cerium precipitates generated nonenzymatically (by addition of exogenous H_2O_2 to the cerium-containing medium) differ in appearance from those generated in the presence of substrate (3). These findings suggest that the cytochemical results are enzyme mediated. In addition, Briggs et al. (3) have shown that formation of cerium precipitates depends on a hydrogen peroxide generating enzyme system since inclusion of catalase into the medium prevented NADH-stimulated formation of reaction product. Biochemical data show that DAO from PMNs is not inhibited by 1 mM KCN (6). Treatment of PMNs with 1 mM KCN in this study revealed no detectable inhibition of enzyme activity based upon formation of cerium reaction product.

The ability of L-alanine to serve as substrate and the localization of reaction product similar to that seen when D-amino acids were used as substrate suggest that both D- and L-amino acid oxidases are present in PMNs and have a similar localization. This demonstration of L-amino acid oxidase activity is supported by Eckstein et al. (11), who have shown that PMNs are capable of oxidizing L-amino acids.

The most important finding was that certain bacteria can stimulate the formation and subsequent detection of sites of H_2O_2 production when incubated in medium lacking exogenously supplied D-amino acids. A gradation in amount of reaction product was observed with the different bacteria employed. Furthermore, the cytochemical results are congruent with the biochemical results using the same bacteria as substrate for purified pig kidney DAO (6). This suggests that DAO may be of some importance in generating H_2O_2 after phagocytosis of some types of bacteria (those with high levels of available cell wall D-amino acids). Localization of reaction product at sites of H_2O_2 production was similar when either inert PS or bacteria were phagocytosed.

The reason for the large percentage of cells which show no DAO activity is not clear; however, this is similar to the cytochemical results for localization of NADH oxidase activity in normal PMNs (4). These authors suggest that the relatively low percentage of active cells may be an accurate reflection of the population of circulating PMNs, i.e., the unreactive cells may be immature, since it is not known when NADH oxidase is produced or active in the life history of PMNs. Alternatively, these results may be artifactual; either the plane of section does not pass through sites of reaction product deposition or the technique lacks the required sensitivity. Which one of these explanations is correct and which one accounts for the results for DAO localization is not known at present.

The ultrastructural localization of DAO to the plasma membrane and phagosome membrane of human PMNs is described in this report. It is

noteworthy that this localization has been achieved with two methods which differ from one another in principle. In one case, a cerium precipitate is formed at the sites of H_2O_2 production. Thus, DAO is detected by localizing H_2O_2, one of the end products of the enzymatic reaction. Similarly, Veenhuis and Bonga (26) have detected DAO in kidney microbodies by this cerium technique. In the second case, DAO in PMNS was localized by use of a ferricyanide technique. In this procedure, ferricyanide serves as an artificial electron acceptor which is reduced to ferrocyanide. Ferrocyanide is subsequently precipitated by Cu^{++} to form the electron-dense copper ferrocyanide precipitate as described by Karnovsky (15). The localization of DAO was essentially the same with either of these techniques. There was one discrepancy, however: DAO could be detected on the surface of resting PMNs with the ferricyanide technique, while reaction product was detected on the surface of PMNs only after phagocytosis with the cerium technique. The reason for this difference is not known, but it may be that the ferricyanide reaction is more sensitive in detecting the localization of DAO than the cerium procedure.

While the general importance of DAO in PMNs has not been resolved, it is of interest that this enzyme has been shown to be on the plasma membrane and can be incorporated into the phagosome membrane during phagocytosis. It has been suggested that the plasma membrane of PMNs can regulate peroxide production (20, 21, 23) or is the site of peroxide production (22). Localization of NADH oxidase to the plasma membrane (3) is direct confirmation that hydrogen peroxide can be generated by a cell-surface component. The present finding that DAO is localized on the cell surface further supports the concept that the plasma membrane is involved in peroxide formation in PMNs.

The authors wish to acknowledge JoAnn Buchanan and Robert Rubin for excellent technical assistance, and Mary Mauri for assisting in the preparation of the manuscript. This research was supported by National Institutes of Health grants HL 09125 and GM 01235.

Received for publication 1 September 1977, and in revised form 19 December 1977.

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