CYTOCHEMICAL DEMONSTRATION OF HYDROGEN PEROXIDE IN POLYMORPHONUCLEAR LEUKOCYTE PHAGOSOMES

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Phagocytosis by polymorphonuclear leukocytes (PMN) is accompanied by specific morphological and metabolic events which may result in the killing of internalized micro-organisms. Hydrogen peroxide is produced in increased amounts during phagocytosis (17) and in combination with myeloperoxidase and halide ions constitutes a potent, microbicidal mechanism (8, 9, 11). There can be direct iodination of micro-organisms (10), or alternatively, other intermediate reaction products, i.e. chloramines and aldehydes (21), can exert a microbicidal effect. The H_2O_2-peroxidase-halide system is presumed to operate within the phagocytic vacuole (12, 18). Myeloperoxidase, present in the primary granules of PMN, enters the phagocytic vacuole during degranulation (1, 4, 7), and halide ions are probably derived from the extracellular medium or are present in the PMN (see 11, 18). For the operation of this system in intact cells, the presence of H_2O_2 in the phagocytic vacuole is necessary, and indeed this has been suggested by the work of several investigators (12, 18, 21). In the present investigation, the diaminobenzidine reaction of Graham and Karnovsky (5), modified to utilize endogenous myeloperoxidase and hydrogen peroxide, has been applied to actively phagocytizing PMN to demonstrate cytochemically the presence of H_2O_2 in the phagocytic vacuole.

MATERIALS AND METHODS

Isolation and Preparation of PMN

A majority of these experiments were performed on human polymorphonuclear leukocytes isolated by a

modification of the technique of Harris (6). A glass cover slip was flooded with blood from a pricked finger, placed in a moist chamber, allowed to stand at room temperature for 5 min, and then incubated at 37°C for 30 min. The blood clot and erythrocytes were rinsed from the cover slip with Hanks' balanced salt solution (Hanks' BSS) (Microbiological Associates, Inc., Bethesda, Md.), pH 7.4, at 4°C, leaving numerous PMN and a few monocytes and eosinophils attached to the glass surface. Additional experiments were performed on suspensions of PMN isolated from rat and guinea pig peripheral blood by Dextran sedimentation, and on guinea pig peritoneal exudate cells produced by injections of sterile 0.9% NaCl. All cell preparations were washed twice with Hanks' BSS before use.

Preparation of Particles for Ingestion

Polystyrene spherules (PS) (1.1-μm diameter, Dow Chemical Company, Midland, Mich.) were dialyzed extensively against twice-distilled H_2O and diluted to 2.5% of the original concentration with saline. Starch particles were prepared from *Amaranthus caudatus* seeds (14). Zymosan (Nutritional Biochemicals Corp., Cleveland, Ohio) was boiled 10 min in saline, washed three times in saline, and opsonized by incubation in autologous serum for 30 min at 37°C (10 mg/ml serum). Particles were washed twice with Hanks' BSS and resuspended in Hanks' BSS (5 mg/ml).

Phagocytosis and Cytochemistry

The cytochemical localization of H_2O_2 was accomplished by using a modification of the diaminobenzidine (DAB) technique (5) designed originally for the localization of peroxidase. The incubation medium contained 0.5 mg DAB (3,3'-diaminobenzidine tetrahydrochloride dihydrate, Aldrich Chemical Co., Inc., Milwaukee, Wis.) per ml of Hanks' BSS with additional glucose (1 mg/ml). Particles were included in excess over cells in the incubation medium at a final concentration of: PS, 1:20 dilution of the 2.5% stock; Zymosan, 1:10 dilution of the 5 mg/ml stock; starch, 0.3 mg/ml. The final pH of the medium was adjusted to 7.3. Cover slip preparations were flooded with 0.5 ml of the medium and incubated at 37°C in a moist chamber for 20 min. In one experiment, cells were incubated in the DAB medium for 10 min, washed with Hanks' BSS, and reincubated for 10 min in Hanks' BSS plus glucose. Cells in suspension were adjusted to a final concentration of $2–2.7 \times 10^6$ cells/ml of incubation medium and maintained at 37°C with constant, gentle agitation for 20 min.

Controls

Controls consisted of omission of DAB or the particles from the incubation medium, or the inclusion of 2 mM KCN, 10 mM 3-amino-1, 2, 4,-triazole, or 0.2 mM sodium azide in the incubation medium. When an inhibitor was used, the cell preparation was preincubated for 10 min at 38°C with the inhibitor in Hanks' BSS, drained, and reincubated in the test medium containing the inhibitor.

Postincubation Processing

After incubation, cells were washed several times with Hanks' BSS at 4°C. Fixation was performed in 2% glutaraldehyde (EM Grade, Polysciences, Inc., Warrington, Pa.) in 0.1 M cacodylate buffer, pH 7.3, with 5% sucrose at 4°C for 60 min. Cells were washed overnight in the same buffer at 4°C, postfixed for 1 h at 4°C in 2% OsO_4 in 0.1 M cacodylate buffer with 5% sucrose, pH 7.3, dehydrated in graded ethanols, and embedded in Epon 812 (13).

Thin sections prepared with an LKB microtome equipped with a diamond knife were examined either unstained or after staining with 4% aqueous uranyl acetate and alkaline lead (24) (3 and 4 min, respectively). Material was examined in a Philips 200 electron microscope operated at 60 kV.

RESULTS AND DISCUSSION

The technique used in this investigation was originally designed for the ultrastructural localization of the peroxidase enzyme which, in the presence of exogenously supplied H_2O_2, oxidizes DAB to an insoluble, osmiophilic polymer, easily visualized in the electron microscope. As applied here, only DAB is supplied, and the generation of reaction product depends upon the presence of endogenous enzyme and H_2O_2. Myeloperoxidase, present in the primary granules of PMN, is discharged into the phagosome (1, 4, 7); hence, the deposition of oxidized DAB-reaction product is indicative of the presence of endogenous H_2O_2. Live, unfixed PMN were used in all experiments and were incubated under relatively physiological conditions (Hanks' BSS, pH 7.3, 37°C) which allowed for apparently normal production of H_2O_2 and delivery of myeloperoxidase to the phagosome. Exposure to particles and to DAB simultaneously permitted entry of DAB into the phagosome. This compound, which has a slight positive charge, presumably binds to polystyrene particles which have a negative surface charge at physiological pH (23). In addition, a very small amount of medium containing DAB is probably ingested with the particles (3).

Examination of PMN exposed to DAB and a phagocytizable particle revealed the presence of electron-dense reaction product within the phagocytic vacuole (Fig. 1); such material is absent in control preparations (Fig. 2). The origin of the PMN (guinea pig, rat, or human peripheral blood,

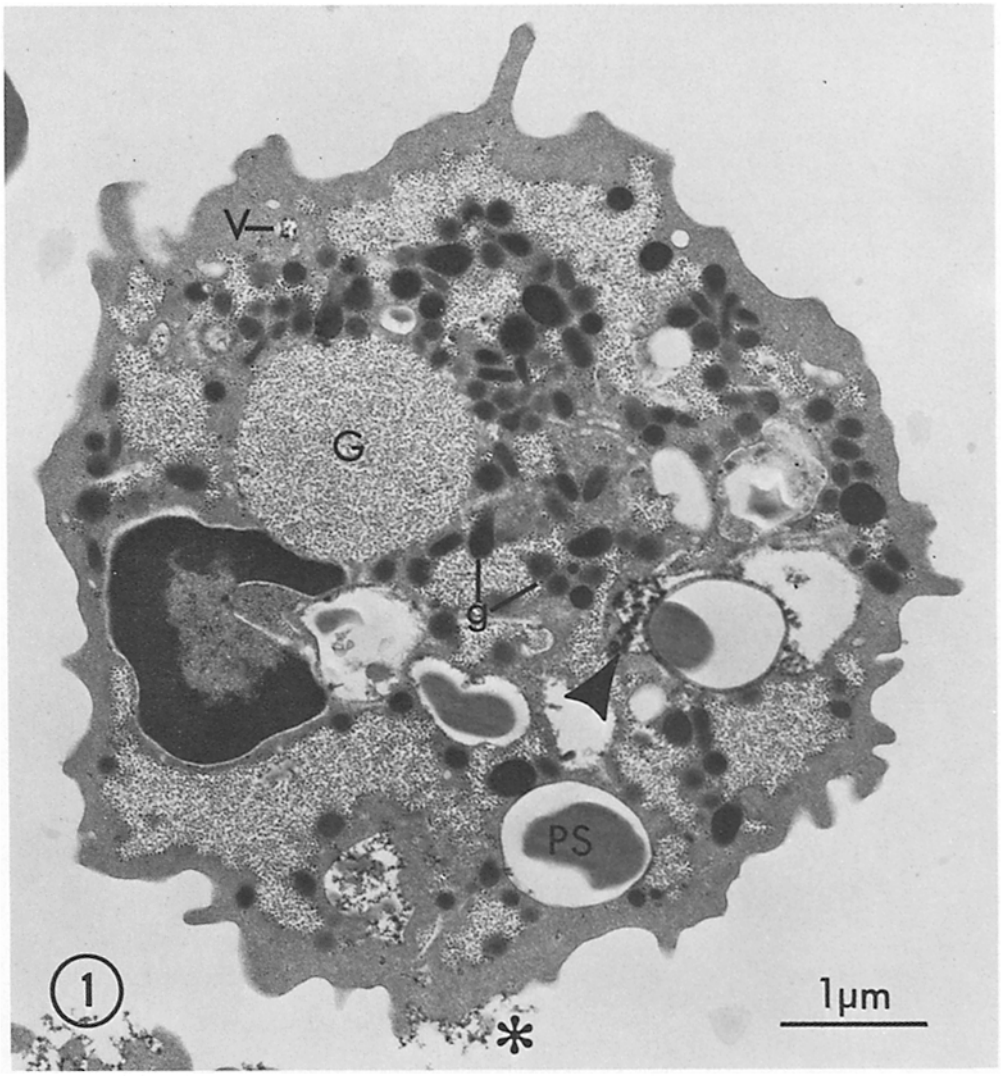

FIGURE 1 PMN from guinea pig exudate exposed to polystyrene (*PS*) and diaminobenzidine for 20 min. Oxidized DAB is present in the phagocytic vacuole (arrowhead) between the phagosomal membrane and the particle. (The polystyrene is partially extracted during preparation for electron microscopy). Additional reaction product is found in occasional small vesicles (*V*) and adsorbed on to the surface of the cell (*). Large deposits of glycogen (*G*) and numerous, dark-staining granules (*g*) are other notable features. These granules (*g*) are not peroxide-positive, for control preparations (without DAB) have similar staining properties (see Fig. 2). × 19,400.

or guinea pig peritoneal exudate), and the type of particle (polystyrene, starch, or opsonized Zymosan), did not alter the results, although more reaction product was found with PS, presumably because more DAB was taken in due to surface binding. Reaction product was located in the space between the particle and the membrane of the phagocytic vacuole, and deposits frequently appeared to be focused in one or two regions, perhaps indicating the point of fusion of primary granules and the phagocytic vacuole (Fig. 3). Occasionally, reaction product was more evenly distributed around the particle. Additionally, negative phagocytic vacuoles were found, presumably because the plane of the section did not pass through the locale of the reaction site or because the H_2O_2 and/or

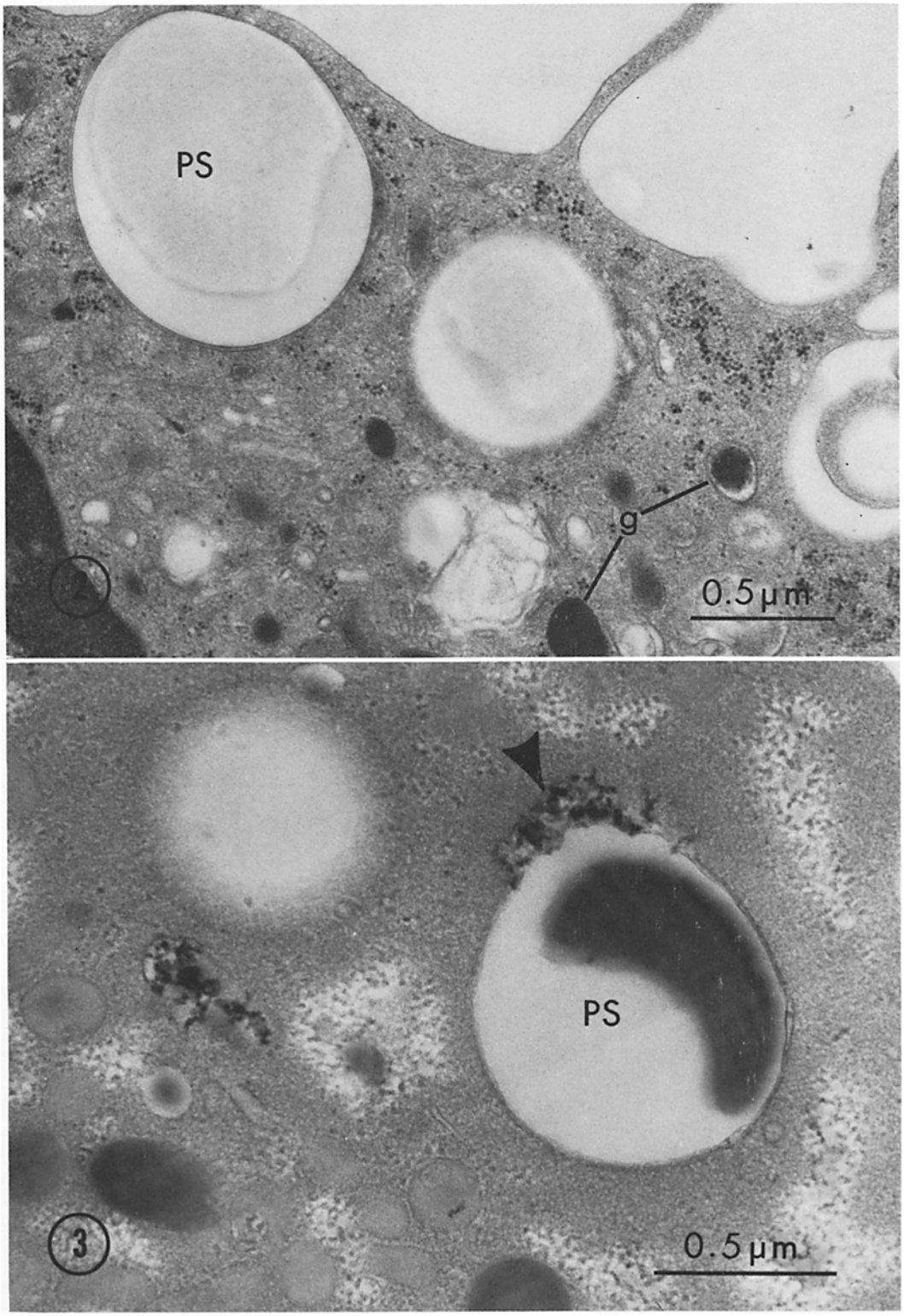

FIGURE 2 Control preparation of guinea pig exudate PMN allowed to phagocytize polystyrene (*PS*) in the absence of DAB. Note that the phagocytic vacuole lacks material comparable in electron density to oxidized DAB. × 44,400.

FIGURE 3 Portion of guinea pig PMN cytoplasm showing a typical focal deposit of reaction product in the phagocytic vacuole. This may represent the site of primary granule fusion and hence the site of the myeloperoxidase acting on endogenous H_2O_2. Experimental conditions as for Fig. 1. × 55,400.

myeloperoxidase had not yet entered the phagocytic vacuole. The absence of myeloperoxidase-dependent reaction product in some phagocytic vacuoles has been previously noted by Bainton (2).

Oxidized DAB was also observed within membrane-limited channels connecting the phagocytic vacuole with the surface of the cell (Fig. 4), as well as in cytoplasmic vesicles (Fig. 1) and adsorbed to the outside of the plasma membrane (Fig. 5). The source of these deposits has not been completely defined. The technique does not permit discrimination between leakage of oxidized DAB and leakage of myeloperoxidase and H_2O_2 from incompletely closed phagocytic vacuoles. It has been shown biochemically (1) and cytochemically (7) that peroxidase is released from phagocytizing PMN, as is hydrogen peroxide (17, 19), so it is conceivable that reaction product can form extracellularly. The presence of reaction product in the vesicles might be attributed to internalization after surface adsorption. This is suggested by experiments in which PMN are briefly (10 min) exposed to PS and DAB, washed well, and then reincubated in Hanks' BSS for 10 min. The washing step was included in the sequence to remove extracellular DAB, PS, peroxidase, and oxidized DAB and resulted in an obvious reduction of adsorbed and vesicular reaction product. However, in this experiment there was no diminution of reaction product in phagocytic vacuoles, indicating that such reaction product was generated *in situ* and not carried in with the particles. Furthermore, extracellular reaction product does not appear to stick to uningested or partially ingested particles. The

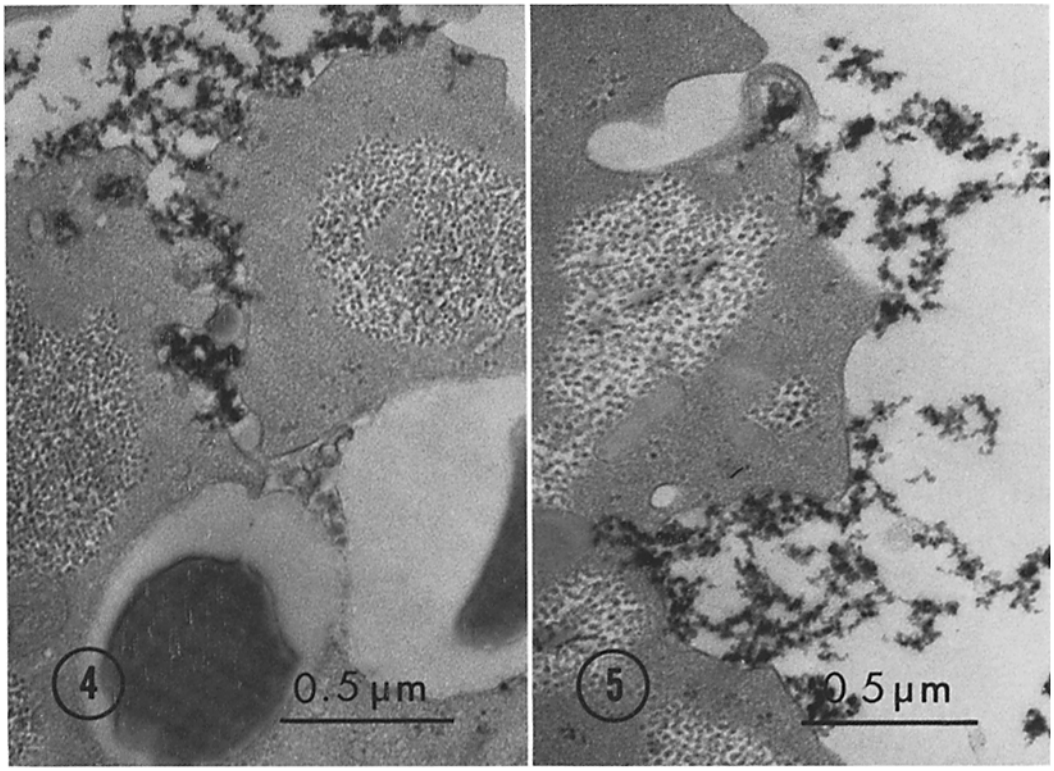

FIGURE 4 Portion of guinea pig PMN cytoplasm taken after a 20-min exposure to DAB and PS. Incomplete closure of the phagocytic vacuole resulting in a surface-connected channel is shown. The presence of reaction product within this channel implies a leakage of myeloperoxidase and H_2O_2, or possibly reaction product, from the phagocytic vacuole. × 46,000.

FIGURE 5 Extracellular reaction product (due to leakage from incompletely closed phagocytic vacuoles) is absorbed on to the plasma membrane where reinternalization may occur. Specimen preparation as for Fig. 1. × 47,100.

peroxidase-containing primary granules are darkly stained (Figs. 1 and 3), but this density is not due to the presence of reaction product because granules in control preparations (Fig. 2) show similar staining properties. Either H_2O_2 or, more probably, DAB fails to reach the granules.

PMN allowed to ingest particles alone did not contain material in the phagocytic vacuoles that was comparable in density to oxidized DAB (Fig. 2), nor did cells appear to take up or oxidize DAB in the absence of particles. Inhibitors of myeloperoxidase activity, azide, aminotriazole, and cyanide, blocked the formation of intracellular and extracellular reaction product, indicating the role of the enzyme in the reaction. Inhibitors did not appear to interfere substantially with phagocytosis.

Since hydrogen peroxide acts as the substrate for myeloperoxidase in the oxidation of diaminobenzidine, these observations provide direct evidence for the presence of hydrogen peroxide in the phagocytic vacuole. This is in keeping with its proposed role in the oxidative microbicidal activity of the PMN (11) and supports the observations (9) which indicate the need for close temporal proximity of the reactants of the myeloperoxidase-H_2O_2-iodide system to the micro-organisms for effective microbicidal activity. The investigations of Root and Stossel (18) on myeloperoxidase-mediated iodination have also implied the presence of H_2O_2 in phagocytic vacuoles.

Hydrogen peroxide synthesis in polymorphonuclear leukocytes depends upon oxidation of reduced pyridine nucleotide(s) (NADH oxidase [7] or NADPH oxidase [20]). The resulting hydrogen peroxide accumulates in the phagocytic vacuole. Due to the diffusibility of hydrogen peroxide within the cells and the required presence of myeloperoxidase to produce the cytochemical reaction product, the results presented here do not necessarily imply that peroxide is generated within the phagocytic vacuole. The site of synthesis of hydrogen peroxide is problematic. NADPH oxidase appears to be associated with a granule fraction of PMN (16), while the subcellular localization of NADH oxidase remains unclear. Some investigators have implied that the plasma membrane, which ultimately forms the phagosome membrane, regulates peroxide production (19, 20), is the site of peroxide production (18, 22), or is the site of a reduced pyridine nucleotide oxidase (15). Research is currently underway to determine, cytochemically and biochemically, the location of NAD(P)H oxidase and the involvement of plasma and phagosome membrane in hydrogen peroxide production.

SUMMARY

The presence of hydrogen peroxide within the phagocytic vacuole of polymorphonuclear leukocytes has been demonstrated by application of the diaminobenzidine technique, modified to utilize endogenous myeloperoxidase and peroxide.

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