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Superoxide production by polymorphonuclear leukocytes

A cytochemical approach

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Summary. Phagocytosis by polymorphonuclear leukocytes triggers a burst of oxidative metabolism resulting in hydrogen peroxide and superoxide production, and these active oxygen species function in the killing of microorganisms. A new cytochemical technique, based on a manganese dependent diaminobenzidine oxidation, has been developed to detect superoxide in these cells. It has been shown that superoxide generation is associated with the plasma membrane in cells activated by particulate (zymosan) and non-particulate (phorbol myristate acetate) stimuli. This membrane activity is maintained during invagination such that reduced oxygen is generated within the endocytic vacuoles. Reaction product is absent from unstimulated cells; additionally, formation of precipitate is blocked by omission of Mn^{++}, low temperature, glutaraldehyde prefixation, and the presence of superoxide dismutase in the incubation medium.

Introduction

The polymorphonuclear leukocyte (PMN) is a phagocytic white blood cell with the primary function of host defense. In this capacity, the cell must recognize and bind foreign particulate material. This attachment of a particle to the surface of the cell initiates internalization and a series of events which result in the killing (in the case of live microorganisms) and destruction of the phagocytosed material. One of these events is an increased metabolic activity or "respiratory burst" which is triggered by perturbation of the cell plasma membrane through contact with the particle (or alternatively by a variety of soluble agents) and is comprised of an increase in hexose monophosphate shunt activity and concomitant cyanide insensitive oxygen consumption. The result is the production of several active (i.e., reduced or excited) oxygen species. The phagocytic leukocytes are capable of generating substantial amounts of superoxide (O_2^-) (Babior et al. 1973) as well as hydrogen peroxide (H_2O_2) which may then interact to form singlet oxygen ($^1O_2$) and the highly reactive hydroxyl radical ($OH\cdot$) (for review, see Babior 1978; Badwey and Karnovsky 1980). The PMN has several killing mechanisms available, and generally they result from the accumulation of microbicidal agents and reactants within the phagocytic vacuole. Some of these cytocidal reactions are dependent, at least in part, upon the oxygen species provided by the respiratory burst. Probably the major and most thoroughly investigated is the hydrogen peroxide-halide-myeloperoxidase combination (see Klebanoff 1975 and 1980), although peroxide appears to be associated with other killing mechanisms as well (Miller 1969; Drath and Karnovsky 1974). Superoxide, the other active oxygen species generated, does not appear to be directly microbicidal (Babior 1978; Klebanoff 1980; VanHemmen and Meuling 1977), but it can dismutate to form H_2O_2 and form singlet oxygen and hydroxyl radicals as indicated above. The latter species are capable of causing cellular damage, but the extent of their roles in the microbicidal activities of the cell remains to be determined (see Badwey and Karnovsky 1980).

The plasma membrane of the PMN is now recognized as having an integral role in all these activities. Not only is it the site of recognition for a number of stimulating agents (particulate and non-particulate), but it is also apparently the site of the activator or trigger of the metabolic burst. Goldstein et al. (1977) and Dewald et al. (1979) have shown biochemically that the site of O_2^- production in activated neutrophils is the plasma membrane and the phagosomal membrane (see also Nathan et al. 1969). Hydrogen peroxide has also been demonstrated at these sites cytochemically (Briggs et al. 1975a, b) and biochemically (Root and Metcalf 1977). The cytochemical study of neutrophil function, especially oxidative cytochemistry, has provided valuable information on the phagocytic process (for review, see Karnovsky et al. 1981 and 1982). In this regard we have now devised a cytochemical method for the demonstration of subcellular sites of O_2^- production in activated neutrophils in addition to the earlier method for H_2O_2 mentioned above. The technique depends on the oxidation of diaminobenzidine (DAB) to an insoluble, osmiophilic polymer. In brief, the cells are exposed to a cytochemical medium containing a peroxidase inhibitor (to avoid interference by H_2O_2), DAB and Mn^{++}. Superoxide (or a derivative) presumably oxidizes the manganous ions to Mn^{+++} (Curnutte et al. 1976; Patriarca et al. 1975), the latter having the ability to oxidize the DAB, resulting in the deposition of electron dense reaction product. Activated PMN accumulate this reaction product in zymosan induced phagocytic vacuoles, in phorbol myristate acetate (PMA) induced vacuoles, and, to a lesser extent, on the external surface of

In honour of Prof. P. van Duijn

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the plasma membranes. A portion of this work has appeared in abstract form (Briggs et al. 1983).

Materials and methods

Cell preparation. Human polymorphonuclear leukocytes (PMN) isolated by a modification of the technique of Harris (1952) were used for all experiments. Clean coverslips (22 mm diameter) were flooded with blood from a finger puncture, placed in a moist chamber, and incubated for 5 min at room temperature followed by 30 min at 37° C. This allowed time for the neutrophils to settle and adhere to the glass and for the serum to clot. The clot was removed and other nonadherent material was flushed from the surface by swirling the coverslip in ice-cold 0.1 *M* Hepes (Sigma Chemical Co., St. Louis, MO) – NaOH buffer, pH 7.2, containing 5% sucrose. This resulted in a monolayer of cells, approximately 90% PMN, remaining attached to the coverslip.

Cell activation. Two approaches were taken to turn on the respiratory burst of the isolated PMN. Cells were induced to phagocytize particles by flooding the coverslip with a suspension of opsonized zymosan (0.5 mg/ml) in 0.1 *M* Hepes buffer, pH 7.2, containing glucose (1.0 mg/ml) and 1 m*M* sodium azide. Following a 5 min incubation at 37° C, uninternalized particles were rinsed away with Hepes-azide buffer; coverslips were then ready for immediate incubation in the cytochemical medium. The zymosan (1 mg/ml) (Sigma Chemical Co., St. Louis, MO) was opsonized just prior to use by a 20 min incubation at 37° C in human serum.

Non-particulate stimulation was accomplished by brief exposure to phorbol myristate acetate (PMA, Sigma Chemical Co., St. Louis, MO). A stock solution of 2 mg/ml DMSO (Fisher Scientific, Medford, MA) was diluted a thousand-fold with 0.1 *M* Hepes buffer, pH 7.2, containing 1 m*M* sodium azide and 5% sucrose just prior to use. Monolayers of cells were flooded with the PMA solution for 90 s at 37° C, then rinsed for 5 min at room temperature in several changes of the same buffer. This was followed immediately by cytochemical testing.

Comparable control (unstimulated) coverslips were prepared by similar incubations in media from which either the opsonized zymosan or the PMA had been omitted.

Cytochemistry. Unfixed cells, activated or resting, were preincubated for 5 min in Hepes buffer with 5% sucrose and 1 m*M* azide; in the case of PMA exposed cells, this was accomplished by the post-stimulation wash. The final cytochemical medium consisted of this same buffer to which had been added 3,3′-diaminobenzidine (DAB, Sigma Chemical Co., St. Louis, MO) (1.0 mg/ml) and 0.5 m*M* Mn^{++} ($MnCl_2 \cdot 4H_2O$). It was necessary to readjust the pH of the medium after adding the DAB, 0.2 N NaOH being used to return it to 7.2. The coverslip preparations were flooded with this medium and incubated at 37° C. The cytochemical medium was prepared just before use, and its stability beyond several hours was not determined. A range of incubation times was tested and optimum times selected to allow for maximum accumulation of reaction product without too much damage to the fine structure of these unfixed cells. Longer incubation times led to the accumulation of more reaction product, but the cell morphology suffered, and the precision of localization was diminished. PMN stimulated with zymosan were usually incubated for 20–30 min, while PMA activated cells were exposed to the cytochemical medium for only 5 min. On occasion, stimulation with PMA was done concurrently with the cytochemistry; PMA was added to the DAB-Mn^{++} medium and cells were treated for 5 min prior to termination.

After the cytochemical incubation, the coverslips were rinsed very briefly in 0.1 *M* Hepes buffer, pH 7.2, with 5% sucrose and 1 m*M* azide at room temperature prior to fixation.

Electron microscopy. The rinsed monolayers were fixed in 2% glutaraldehyde (E.M. grade, Polysciences, Warrington, PA) in 0.1 *M* cacodylate buffer, pH 7.3, with 5% sucrose at 4° C for 30–60 min. All cells were washed overnight at 4° C in several changes of buffer and postfixed in 2% OsO_4 in the same buffer for 60 min at room temperature. The cells were dehydrated through a graded series of ethanols followed by two 5 min changes in propylene oxide, then infiltrated with Epon 812. For embedding, Beem capsules without covers were filled with Epon, inverted over the cells on the coverslip, and polymerized at 60° C for 24 h. A clean separation of Epon blocks containing the cells from the coverslips occurred when they were briefly immersed, while still warm, in liquid nitrogen. The monolayer of cells was found on the face of the block. Thin sections were cut on an LKB III or V Ultrotome and examined either unstained or stained with 4% aqueous uranyl acetate and lead citrate in a Philips 200 electron microscope operated at 60 kV.

Controls. A number of different controls were run in order to ascertain the specificity of the reaction and the dependability of the localization. These will be discussed at appropriate points in the Results.

Results

Superoxide detection by DAB-Mn^{++}

It has been proposed that superoxide, through a nonenzymatic free-radical chain of reactions, can cause the oxidation of NADPH in the presence of manganese (Curnutte et al. 1976; Patriarca et al. 1975). This reaction is brought about through the oxidation of Mn^{++} to Mn^{+++} and the subsequent oxidation of NADPH by the highly reactive Mn^{+++}. The cytochemical technique described here is thought also to depend on the oxidative nature of the Mn^{+++}, and the reaction has been modified such that the final electron donor is the artificial substrate DAB. The proposed cytochemical reaction was found to occur at near neutral pH in 0.1 *M* Hepes-NaOH buffer, a buffer which appears suitable also for preservation of enzyme activity and subcellular integrity. Azide is included in the medium to block endogenous peroxidatic activity which might otherwise cause DAB precipitation.

Superoxide localization

Live, unfixed PMN which had been exposed to opsonized zymosan prior to incubation in the DAB-Mn^{++} medium showed a characteristic and reproducible distribution of reaction product. It was found on the external surface of the plasma membrane and on the luminal surface of the membrane of the phagocytic vacuole (Fig. 1). The outer surface location was rather variable in that some cells exhibited fairly abundant reaction product while others showed reduced amounts in small, isolated patches. Involvement of the entire surface was uncommon, and there were many cells showing a plasma membrane profile devoid of detectable reactivity. When oxidized DAB was found within the phagocytic vacuoles, it was sandwiched between the membrane and the zymosan particle within. If particles were only partially internalized, reaction product was seen between the particle and the presumptive phagocytic vacuole membrane, and the channels connecting incompletely closed phagosomes to the surface were often lined with precipitate. As with the surface, not all the phagosomes were reactive, nor was the entire inner surface necessarily reactive. These phagocytic cells also frequently contained small vesicles and vacuoles scattered in the cytoplasm, and many of these contained deposits; these may be surface infoldings or profiles of channels. No other cellular components or organelles were reactive; zymosan incubated alone

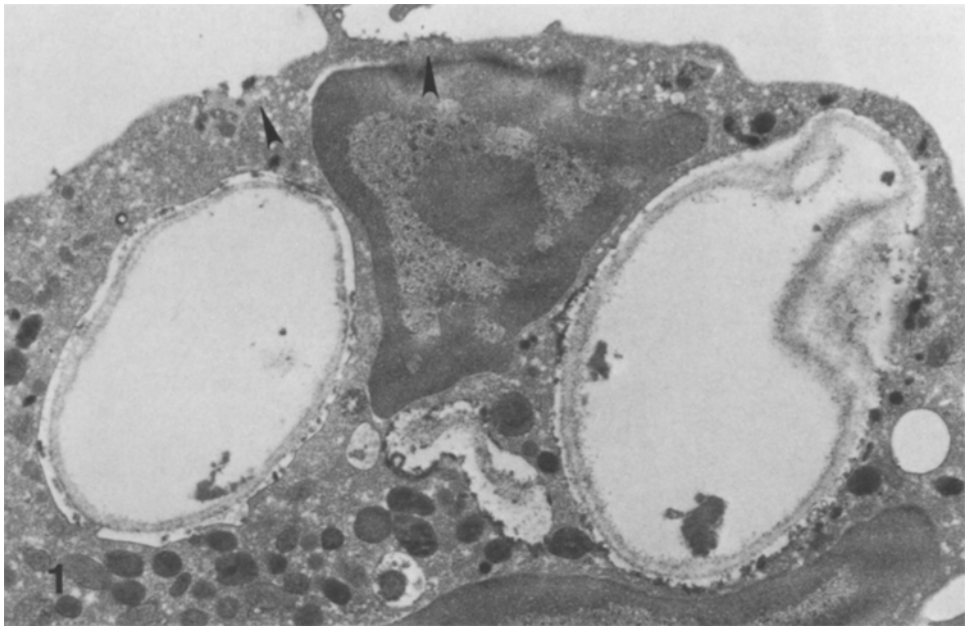

Fig. 1. Zymosan stimulated polymorphonuclear leukocytes (PMN). Reaction product is found within the phagocytic vacuole, between the particle and the surrounding membrane. The plasma membrane is less frequently and less extensively involved; when precipitate is present, it is on the external surface (*arrowheads*). ×14,800

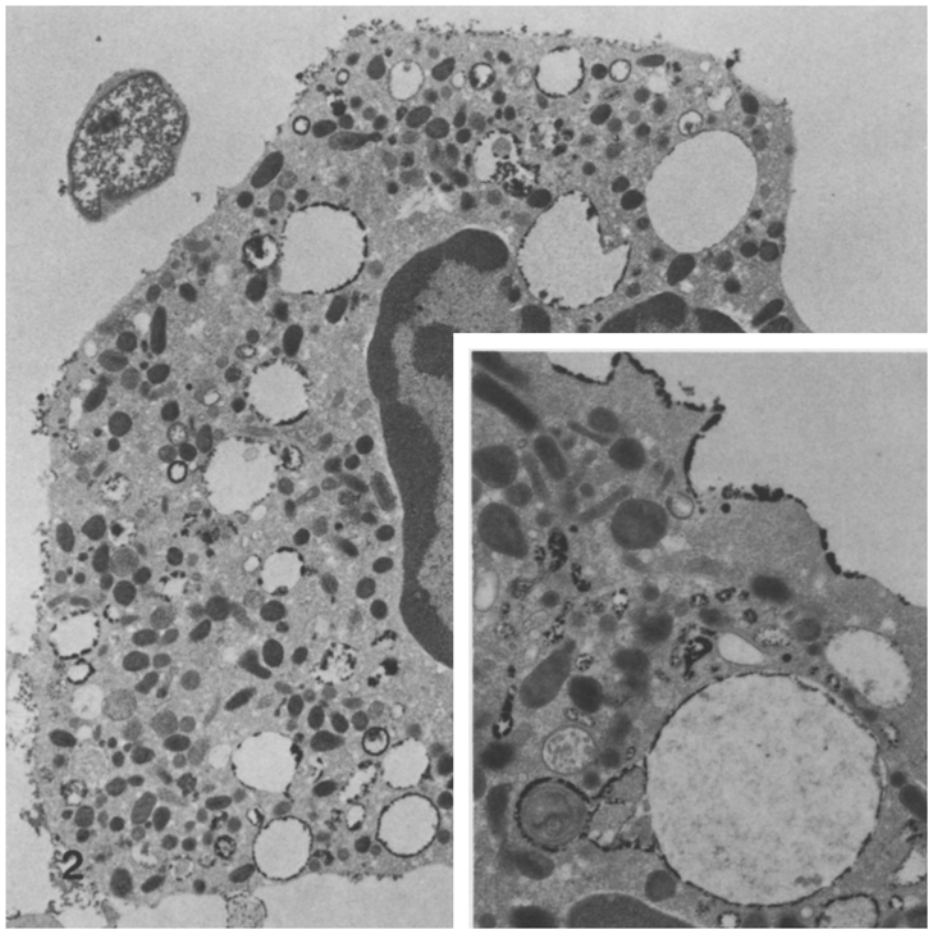

Fig. 2. Phorbol myristate acetate (PMA) stimulated PMN. These cells exhibit a characteristic and reproducible distribution of reaction product. Almost all of the PMA-induced cytoplasmic vacuoles show precipitate on the luminal surface. Similar deposits are present, to a more variable extent, on the external surface of the plasma membrane. The remainder of the cell is unreactive. *Inset:* a higher magnification of a PMA treated cell, showing surface and vacuolar reactivity. ×10,000; *inset*, ×21,400

in the complete medium was negative. There was always a range of reactivity within the phagocytically active monolayer population. Some cells were very reactive, others entirely unreactive within the cross-sectional profile; the majority fell somewhere in between.

Exposure of PMN to PMA alters the morphology of the resting cell by introducing a large number of intracellular vacuoles, presumably of cell surface origin. These are highly variable in size and are generally most abundant around the cell perimeter. A majority of these PMA induced

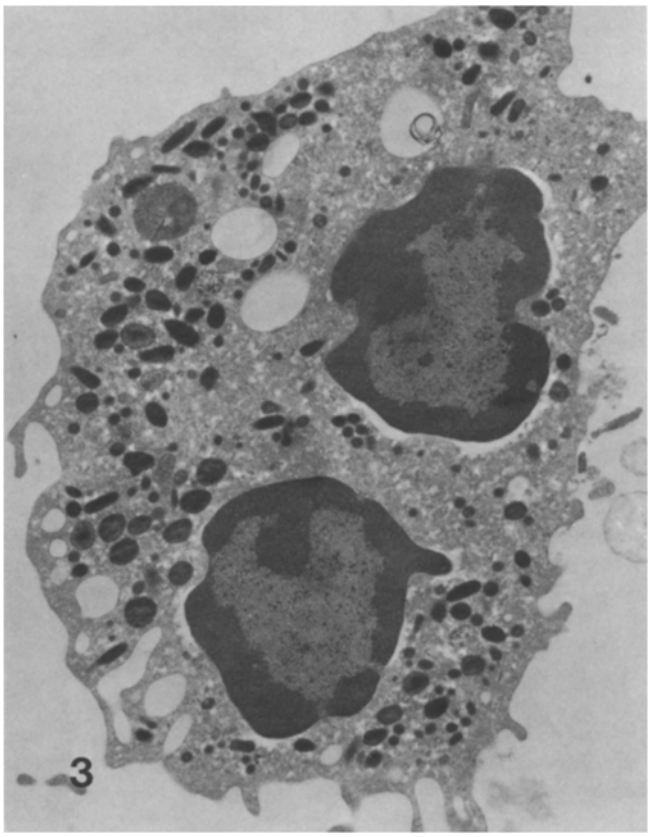

Fig. 3. Unstimulated cell. A resting PMN shows no sign of cytochemical reactivity associated with the membrane or other structures. ×9,900

vacuoles, regardless of size, consistently show the presence of reaction product associated with the luminal surface of the membrane (Fig. 2 and inset). Electron-dense precipitate was also variably present on the surface of the cell. In some cases nearly the entire perimeter was reactive, in others only discreet localized regions. Again, reaction product was found in only these two areas; the remainder of the cell was negative. As evidenced by precipitate generation, PMA was found to activate almost all the PMN in the monolayer, there being relatively few unreactive cells. As a result of this reliability, PMA was the stimulus of choice for most of the control experiments.

Controls

As the initial control for zymosan or PMA activated cells, unstimulated, resting PMN (live and unfixed) were incubated in the DAB-Mn^{++} medium and only occasionally were electron-dense deposits visible in small cytoplasmic vacuoles (Fig. 3). An occasional cell was found to have more reactivity, and these were often observed to have phagocytized some debris. Again, no cytoplasmic organelles showed electron-dense deposits.

A number of different approaches were taken to assess the specificity of the reaction and the dependability of the localization. All of the controls to be reported were performed on PMA stimulated cells, since this approach was faster, more stringent and dependable, and gave a much higher percentage of cells containing reaction product; similar results were found on zymosan-stimulated cells.

1. Chemistry of the cytochemical reaction: Activated cells incubated in the cytochemical medium lacking either DAB or Mn^{++} (Fig. 4) failed to show any reaction product, either on the surface or within the vesicles or vacuoles. This was true even after prolonged incubations (up to 60 min).

2. Effect of temperature: Rinsing and incubating PMA stimulated cells in the complete cytochemical medium at 4° C instead of 37° C resulted in either a lack of reaction product formation or, as shown by an occasional cell, very small deposits of reaction product.

3. Aldehyde prefixation: Aldehydes are known to inhibit the activity of some enzymes. PMA stimulated cells were fixed in 2% glutaraldehyde in 0.1 *M* cacodylate buffer, pH 7.2, with 5% sucrose at 4° C for 15 min followed by a 15 min rinse in 0.1 *M* Hepes-azide, pH 7.2, and subsequent incubation in the complete cytochemical medium. This resulted in a total elimination of reaction product from the cells. A less drastic approach involved fixation of PMA activated PMN in 1% formaldehyde (prepared from freshly depolymerized paraformaldehyde, Mallinckrodt Chemical Co., St. Louis, MO.) in 0.1 *M* Hepes-azide (pH 7.2) at 4° C. This fixation schedule resulted in only a slight diminution in the amount of the reaction product and no change in the sites of deposition (Fig. 5). Controls were stimulated, then rinsed for 30 min in Hepes-azide at 4° C prior to incubation and presented normal amounts of precipitate. Apparently superoxide generation is more sensitive to dialdehyde fixation, presumably by inhibition of enzyme activity. Paraformaldehyde prefixation, less inhibitory in its effects, also gives the assurance that the reaction product on the surface and surface derived endomembranes of unfixed cells is formed at the sites visualized, for this prefixation diminishes the possibility of postprecipitation rearrangements.

4. Peroxidase inhibitors: It has been well documented that myeloperoxidase and catalase are found in PMN, and these enzymes in the presence of DAB and endogenous hydrogen peroxide are capable of DAB oxidation (see Briggs et al. 1975a). To preclude this potential interference with the specificity of the DAB-Mn^{++} reaction for superoxide, the incubation medium routinely contained 1 m*M* sodium azide (or 10 m*M* 3-amino-1,2,4-triazole, AT, Aldrich Chemical Co., Milwaukee, WI). These are peroxidase inhibitors and are reported to have no effect on the respiratory burst, hydrogen peroxide production or superoxide production (Babior et al. 1976). Omission from the incubation medium might allow for increased amounts of reaction product within the vacuoles and extracellularly, as shown by Briggs et al. (1975a), but the effectiveness of the azide inhibition was verified when stimulated cells were incubated in medium without Mn^{++}; no DAB oxidation occurred. A third inhibitor, KCN, was tried for it too is a peroxidase inhibitor. Also, quite importantly, one of the key characteristics of the respiratory burst is its cyanide insensitivity (Sbarra and Karnovsky 1959); superoxide production is likewise cyanide insensitive (Babior et al. 1976). Inclusion of 1 m*M* KCN in the incubation medium allowed for the formation of reaction product in the characteristic locations, but the appearance of the precipitate was slightly altered; in some areas it formed larger and thicker aggregates on the membrane. Cohen et al. (1980) noted cyanide stimulated superoxide production in guinea pig cells, and perhaps an in-

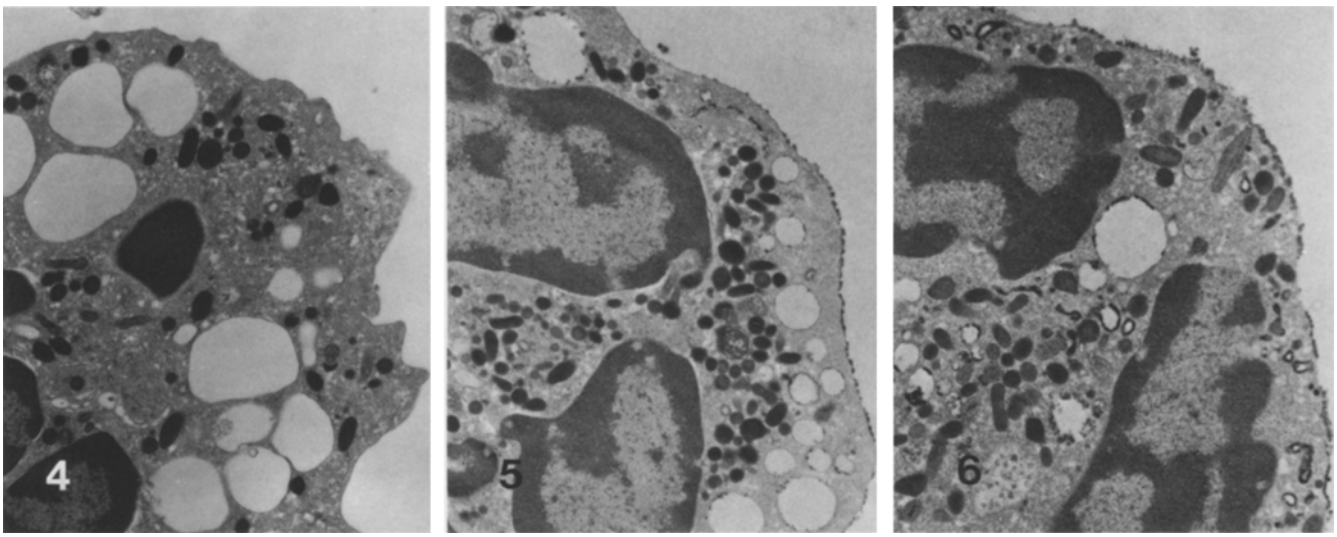

Fig. 4. Medium without Mn^{++}. PMA activated cells, incubated in a cytochemical medium lacking Mn^{++}, fail to show any reaction product. A similar negative result was found when DAB was omitted. × 10,800

Fig. 5. Prefixed cells. Brief fixation in paraformaldehyde prior to cytochemical incubation has essentially no effect on the distribution of precipitate on PMA stimulated cells, the vacuoles and the plasma membrane being the only reactive sites. × 9,300

Fig. 6. Catalase effect. Inclusions of catalase in the cytochemical medium (without azide) has no effect on the amount or distribution of oxidized DAB in PMA activated PMN. × 10,100

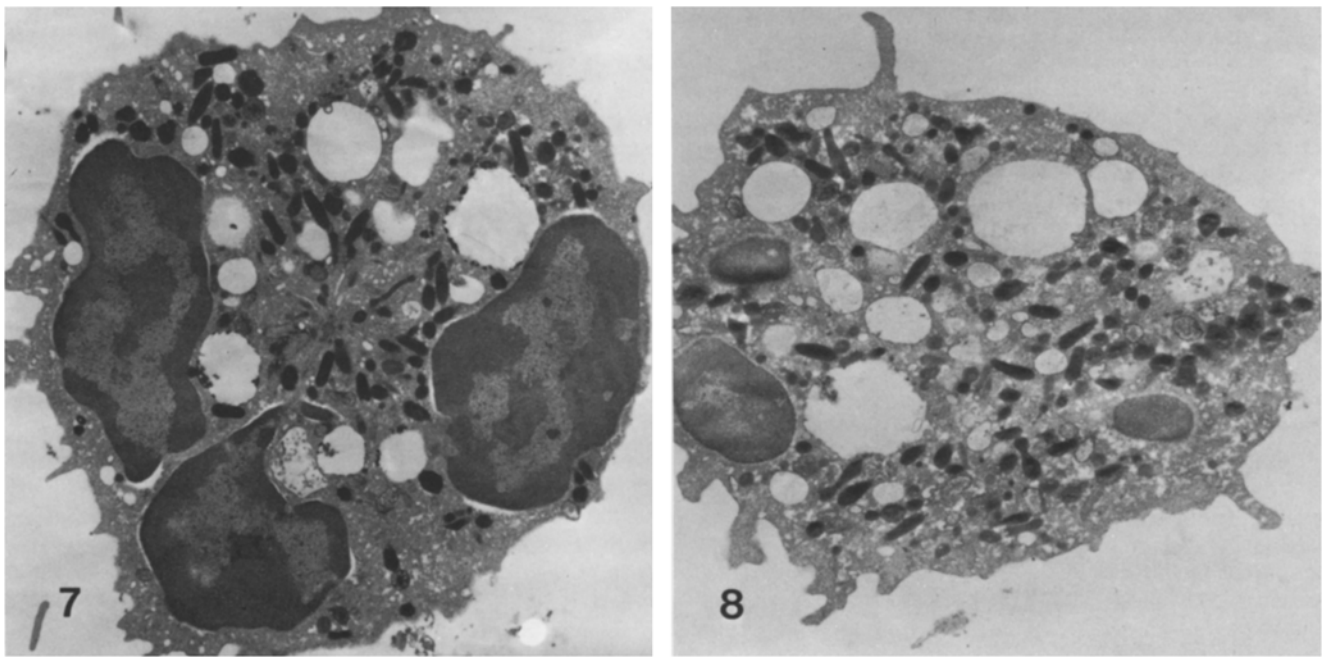

Fig. 7. Superoxide dismutase (SOD) effect. Support for the contention that superoxide is involved in the cellular reactivity comes from the effect of SOD. When SOD is included in the cytochemical medium, PMA stimulated PMN show no reactivity on the plasma membrane, although many of the induced vacuoles are positive. × 8,600

Fig. 8. SOD effect. When SOD is included in the PMA activating solution as well as in the cytochemical medium, reactivity on the surface and in the vacuoles is strongly diminished. × 9,400

creased rate is occurring here, resulting in more precipitate. This altered appearance may also have something to do with the reductive nature of the cyanide affecting DAB oxidation. This result was not further explored, nor was KCN used routinely.

5. Effect of catalase: In a further attempt to separate this reaction from a potential peroxide-peroxidase localization, catalase, which enzymatically catabolizes peroxide, was used to prevent peroxide accumulation in the medium (Seligman et al. 1968) and thus determine if reaction product

generation was peroxide dependent. In these experiments, 0.015% catalase (Sigma Chemical Co., St. Louis, MO.) was added to all media, including washes, PMA stimulation solution (to insure its presence within the newly forming, surface-derived vesicles), and the complete cytochemical incubation medium. Azide was omitted in these experiments. The presence of catalase under such conditions had no effect on the amount or distribution of reaction product (Fig. 6).

6. Effect of superoxide dismutase: Superoxide dismutase (SOD) dismutates superoxide into hydrogen peroxide; it is active at near neutral pH (Fridovich 1978) and is not inhibited by azide. To assess the specificity of the reaction, SOD (Truett Laboratories, Biochemical Division, Dallas, TX) was included in the cytochemical medium at a final concentration of 400 units/ml. Both stimuli were examined. Cells which had actively phagocytozed opsonized zymosan were incubated in the DAB-Mn^{++} medium with added SOD, and a large majority of the cells showed no reaction product or very reduced amounts. Most of the phagocytic vacuoles appeared open to the outside, presumably allowing some access of the SOD to the presumptive vacuolar membrane. When PMA was the stimulus, two approaches were used. One was similar to the zymosan trial; after activation, cells were incubated in DAB-Mn^{++} with SOD. In this case the plasma membrane was largely negative, but many of the PMA induced vacuoles were reactive (Fig. 7). If however SOD at the same concentration were added to both the activating solution (so that it is included in the forming vacuoles) and the complete incubation medium, essentially no reaction product was found on the surface and there were many fewer reactive vacuoles (Fig. 8). This strongly sugggests that the oxidized DAB precipitate is due to a reaction with superoxide or its derivative, a reaction that can be interrupted by dismutation of the superoxide.

Discussion

The alterations in the oxidative metabolism of PMN after stimulation by a diversity of soluble and particulate agents have been well documented and include an increase in oxygen consumption and a subsequent generation of activated oxygen species (for review, see Badwey and Karnovsky 1980). One such species is superoxide, and we have examined by a new cytochemical technique the localization of the site of superoxide production in stimulated human PMN. The design of the ultrastructural test is an adaptation of a biochemical test for the Mn^{++} enhancement of NADPH oxidation by NADPH oxidase. This reaction was found to be inhibitable by superoxide dismutase, and subsequent study showed the role of the Mn^{++} to be nonphysiological in nature (Curnutte et al. 1976; Patriarca et al. 1975). It is proposed that the NADPH is oxidized through a 'nonenzymatic superoxide-dependent free radical chain reaction'; superoxide is protonated to form the hydroperoxyl radical ($HO_2^{\cdot}$) which oxidizes Mn^{++} to Mn^{+++}, the latter being responsible for the oxidation of NADPH (Curnutte et al. 1976). It is this oxidative activity of the Mn^{+++} which forms the basis of this cytochemical test, for it appears capable of oxidatively polymerizing 3,3′-diaminobenzidine, resulting in formation of an insoluble, osmiophilic precipitate. Although this is only proposed as the appropriate sequence of events, the fact that omission of Mn^{++} (or DAB) from the incubation medium eliminates reaction product formation reinforces the probability of this pathway, although other possibilities exist.

When live PMN are incubated in the complete cytochemical medium, there is a stimulus dependent, surface-associated reaction product accumulation. This implies that superoxide is produced only by stimulated cells, that it is generated on the external surface of the plasma membrane, and that this activity is maintained by the membrane as it is invaginated to form the phagocytic vacuole.

In the evaluation of this cytochemical technique, it must be demonstrated that reaction product formation is dependent upon the activity of an enzyme system. It has been found that: (a) the amount of precipitate accumulated appears to be proportional to incubation time; (b) incubation at reduced temperature (4° C) inhibits reaction product formation; (c) prefixation in 2% glutaraldehyde, a crosslinking dialdehyde, completely blocks all signs of activity; and (d) reaction product is formed only after the "respiratory burst" of the cell has been triggered. From these observations we conclude that reaction product formation is the result of enzymatic activity, and this activity must be turned on in order to generate the precipitate. Particulate (opsonized zymosan) or soluble (PMA) stimuli, both known to initiate superoxide production, are capable of inducing reaction product formation.

In analysis of the specificity of the DAB-Mn^{++} reaction, one must keep in mind the number of DAB-oxidizing reactions. DAB can be oxidatively polymerized by peroxidases and catalases, with the reduction of H_2O_2 to water (Graham and Karnovsky 1966; Seligman et al. 1968) and has been utilized in the cytochemical study of a variety of peroxidatic reactions. It has been shown using this technique that the primary granules of the PMN contain peroxidase (Bainton and Farquhar 1968) and that this enzyme is released into the phagocytic vacuole when the primary granule fuses with the phagosome membrane (Baehner et al. 1969). A modification of the DAB technique has been used to localize H_2O_2, and in stimulated PMN endogenous peroxidase has been demonstrated within the phagocytic vacuole (Briggs et al. 1975a). It is important to eliminate the possibility that the results presented here show simply a peroxide-peroxidase or catalase reaction. This has been addressed by determining: a) the insensitivity of the test to a variety of peroxidase inhibitors (azide, aminotriazole, and cyanide) and its sensitivity to aldehyde fixation (myeloperoxidase is insensitive to short term glutaraldehyde fixation); b) by the absolute need for Mn^{++} in the reaction sequence (Mn^{++} is not necessary for the traditional peroxidase demonstration with DAB); c) by the fact that reaction product is formed not only within the phagocytic vacuole, but on the surface of the cell, a site not found to be reactive for either endogenous peroxidase (Bainton and Farquhar 1968; Baehner et al. 1969) or endogenous peroxide by the DAB method (Briggs et al. 1975a), and within PMA induced vacuoles known not to contain peroxidase (White and Estensen 1974), and d) by the lack of effect of exogenous catalase, a peroxide scavenger, on reaction product deposition. These observations confirm that neither peroxide nor peroxidase is being demonstrated, but leave open the question of the source of the oxidized DAB. There is both indirect and direct evidence that reaction product formation is dependent upon superoxide or a superoxide product. First of all, it is known that PMN must be stimulated in order

to make superoxide in a cyanide insensitive manner, and we find that with the current system reaction product formation in the PMN is cyanide insensitive and requires stimulation. But by far the most convincing argument with regard to superoxide specificity is the inhibitory influence of superoxide dismutase on reaction product generation. This azide insensitive enzyme rapidly dismutates superoxide into hydrogen peroxide. When included in the incubation medium, reduction or elimination of DAB oxidation was the result. This strongly supports our contention that superoxide (or one of its derivatives) is responsible for the generation of the electron dense precipitate found associated with the plasma and vacuolar membranes of these cells.

The dependability of the localization to the plasma membrane and the phagocytic vacuole of the superoxide generating system must also be verified. A possibility to be excluded is that the precipitate is interalized after formation at the surface and subsequently added to the phagocytic or PMA-induced vacuoles. Additionally, although the site of detection is at the membrane-extracellular space interface, for this is the limit to the penetration of SOD, it might be possible that the superoxide is generated in the interior of the cell, for example, in association with the azurophilic granule. This would mean it must escape the cytoplasmic SOD (Salin and McCord 1974; Johnson et al. 1975; DeChatelet et al. 1975) and diffuse to the surface or vacuole where it would encounter the DAB-Mn^{++} medium. These are unlikely occurrences, for prefixation with paraformaldehyde mitigates against postincubational rearrangements of surface bound precipitate, and certainly the reactants can penetrate the cell membrane, especially a lightly fixed membrane, and thus would be expected to show up cytoplasmic sites of superoxide synthesis were they present. Indeed it is the very fact that superoxide can be detected extracellularly that led Salin and McCord (1974) to propose its production on the external surface and within the vacuoles.

Although it is interesting to ask whether the superoxide forming system is the same as the peroxide forming oxidase demonstrated previously (Briggs et al. 1975a, b), we are unable to answer at this time. It has been proposed that these cells generate superoxide initially and the resultant H_2O_2 is derived from dismutation (Root and Metcalf 1977). Perhaps the cerium technique which localizes peroxide is detecting the end product of dismutation of superoxide, just as the DAB-Mn^{++} technique may be detecting Mn^{+++} generated by $HO_2^{\cdot}$, a radical derived from superoxide interactions. In any case, neither the Ce^{+++} nor the DAB-Mn^{++} procedures directly detects that site of the enzyme itself; both show only the deposition of peroxide or superoxide dependent reaction product on the external surface of the plasma membrane. The enzyme may be on the external face, or it may be arranged within the plasma membrane such that H_2O_2 and/or O_2^- generation on the external face is the result of transmembrane electron shuttling from a cytoplasmic electron donor (see Segal and Jones 1979; Millard et al. 1979; Green et al. 1980; Babior et al. 1981; Borregaard et al. 1983; also McPhail and Snydeman 1984). In cytochemical experiments the surface of both zymosan and PMA stimulated cells appears less reactive to O_2^- than H_2O_2 (see Briggs et al. 1975b; also Badwey et al. 1980). These differences may be reflecting different enzyme activities or cell subpopulations, but more likely they have to do with differences in the techniques. Mn^{++} oxidation is pH dependent, proceeding more rapidly at acid pH, and the latter favors the formation of $HO_2^{\cdot}$, a more powerful oxidant than O_2^- (Curnutte et al. 1976). The acid pH of the unfixed phagosome (Jensen and Bainton 1973), and perhaps similarly the PMA induced vacuole, would favor this reaction over the one going on in the possibly more alkaline pH of the plasma membrane-external medium microenvironment, thus resulting in a greater accumulation of reaction product within the cytoplasmic vacuoles as opposed to the external membrane face.

Nevertheless, based on the utilization of this new cytochemical technique specific for the detection of superoxide, our results support the hypothesis that the superoxide generating system in activated PMN is associated with the plasma membrane (Dewald et al. 1979). This activity is maintained during phagocytosis or membrane invagination so that at least a portion of the reduced oxygen is generated within the phagocytic vacuole where it functions in the destruction of ingested microorganisms.

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