Electron microscopic identification of hydrogen peroxide detected in fixed human polymorphonuclear leukocytes during phagocytosis

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Summary: Polymorphonuclear leukocytes (PMNs) engaged in phagocytosis produce reactive oxygen species (ROS), such as those that occur in an activated NADPH oxidase reaction, to eliminate ingested microorganisms. The translocation of NADPH oxidase components to produce antimicrobial free radicals from the vesicles to the phagosomes may be important. Hydrogen peroxide (H_2O_2) derived from O_2^- has been observed by electron microscopy using a cerium method. However, 2'-7'-dichlorofluorescein diacetate can also detect H_2O_2 through fluorescence. The main objective of the present study was to measure the H_2O_2 -dependent fluorescence of PMNs after opsonized zymosan A (OPZ) phagocytosis using a microplate reader under different fixation conditions, including 0.5, 1, and 10% glutaraldehyde (GA) individually for 1, 5, 10, or 30 min. An additional objective was to visualize, through the use of electron microscopic cytochemistry, the process of H_2O_2 generation in OPZ phagocytic fixed PMNs. The fixed PMNs showed that the largest fluorescent value was produced by a concentration of 0.5% GA for all fixation times. This suggested that the fixation of PMNs with a high concentration of GA inhibited phagocytosis and produced ROS. In the fixed PMNs, electron microscopic results showed that after 1 min of mixing, some PMNs attached to particles and exhibited mild deposits in their secretory vesicles. When PMNs engulfed particles, free radical-producing vesicles had enhanced reaction deposits 10 min later and fused to the phagosomal membrane, releasing numerous free radicals into the lumen. Time-dependent H_2O_2 production was enhanced in the secretory vesicles, some of which were fused exactly to the phagosome membranes.

Introduction

Polymorphonuclear leukocytes (PMNs) engaged in phagocytosis produce reactive superoxide anions (O_2^-) in a stimulated NADPH oxidase reaction (Segal, 2005). In the original studies locating the dormant enzyme (flavocytochrome b_{558}), Borregaard and colleagues (1992; 1993) clearly showed that intracellular secretory vesicles contain alkaline phosphatase, but not myeloperoxidase, lactoferrin, or gelatinase. The translocation of NADPH oxidase components to produce antimicrobial free radicals from the vesicles to the phagosomes may be important. Hydrogen peroxide (H_2O_2) derived from O_2^- has been observed by electron microscopy using a cerium method. Additionally, 2'-7'-dichlorofluorescein diacetate (DCFDA) can also determine H_2O_2 -dependent fluorescence.

The main objective of the present study was to measure the H_2O_2 -dependent fluorescence of PMN after opsonized zymosan A (OPZ) phagocytosis comparing different

fixed conditions, including 0.5, 1, and 10% glutaraldehyde (GA) individually for 1, 5, 10, and 30 min. An additional objective was to visualize, through the use of electron microscopic cytochemistry, the process of H_2O_2 generation in OPZ phagocytic fixed PMNs.

Materials and methods

PMN

PMNs were isolated from peripheral blood of healthy donors by the Ficoll-Hypaque method. The cells were suspended in an ice-cold phosphate buffered salt solution.

Opsonized zymosan

Ten milligrams of zymosan A were added to 1 ml autologous fresh serum and incubated for 30 min at 37°C. OPZ was then centrifuged and washed in phosphate-buffered saline.

Fig. 1. Electron micrographs of PMNs phagocytosing OPZ. H_2O_2 production is visualized by Ce deposits (arrows). OPZ phagocytosis for 10 min $\Rightarrow$ Ce reaction for 20 min $\Rightarrow$ 2% GA fixation for 60 min.

Electron microscopic cytochemistry of living PMNs

Unfixed intact PMNs were mixed with OPZ for 10 min at 37°C and immediately incubated in a cerium medium (1 mM $CeCl_3$, 0.1% glucose, 7% sucrose, 0.1 M Tris-maleate buffer, pH 7.4) for 20 min at 37°C to determine H_2O_2 ^{3,4)}. After the reaction was completed, the recentrifuged cell pellet was mixed in a cold 2% GA solution in 0.1 M cacodylate buffer at pH 7.4 and fixed for 60 min at 4°C. The cells were then refixed in 1% osmium tetroxide in the same buffer for 60 min at 4°C, dehydrated in graded ethanol, and embedded in Quetol 653. Ultrathin sections were examined under an electron microscope (JEM 1210) without uranyl acetate and lead citrate counterstaining.

H_2O_2 -dependent fluorescence of fixed PMN

PMNs were prefixed for 1, 5, 10, and 30 min with 0.5, 1, and 10% concentrations of GA immediately after OPZ phagocytosis for 10 min, but before a 10^{-8} M DCFDA reaction incubated with 3 mM NADPH. H_2O_2 -dependent fluorescence was determined by a platereader (Multilabel-Reader Mithras LB940; Berthold Technologies, Bad Wildbad, Germany). Morphometric measurements were performed in each of the groups. All data are presented as means $\pm$ S.E. Student's t-test was used to determine significance among all groups. Significance was accepted at $P < 0.05$.

Electron microscopic cytochemistry of fixed PMNs

Other PMNs were immediately fixed in 0.5% GA for 10 min at 4°C after OPZ phagocytosis for 1 and 10 min but before the reaction incubation. The reaction medium contained 1 mM $CeCl_3$, 0.1% glucose, 7% sucrose, 0.1 M Tris-maleate buffer, pH 7.4 and 3 mM NADPH for 20 min

Fig. 2. H_2O_2 -dependent fluorescence of PMN determined after OPZ phagocytosis, as compared with different fixated conditions. OPZ phagocytosis $\Rightarrow$ GA fixation with different conditions $\Rightarrow$ DCFDA fluorescence determination. Note the largest fluorescent value.

Fig. 3. Electron microscopic cytochemistry of fixed PMN. Mild deposit production in the secretory vesicle (arrows). OPZ phagocytosis for 1 min $\Rightarrow$ 0.5% GA fixation for 10 min $\Rightarrow$ Ce reaction (+3 mM NADPH) for 20 min.

at 37°C. The cells were then refixed in the same buffer containing 1% osmium tetroxide for 60 min at 4°C, dehydrated in graded ethanol and embedded in Quetol 653. Ultrathin sections were examined under an electron microscope (JEM 1210) without counterstaining.

Fig. 4. Electron microscopic cytochemistry of fixed PMN. Ce reaction-positive secretory vesicles (arrows) fused to the phagosomal membrane. OPZ phagocytosis for 10 min $\Rightarrow$ 0.5% GA fixation for 10 min $\Rightarrow$ Ce reaction (+3 mM NADPH) for 20 min.

Results

Electron microscopic cytochemistry of living PMN

The PMNs were mixed with OPZ for 10 min at 37°C and incubated for 20 min at 37°C in a cerium medium before fixation. Produced H_2O_2 was captured by cerium ions to form cerium perhydroxide deposits (Fig. 1). The reaction product was visible on the phagosomal membranes and on the areas of the plasma membrane adherent to OPZ.

H_2O_2 -dependent fluorescence of fixed PMN

The fixed PMNs showed that the largest fluorescent value was produced by a concentration of 0.5% GA for all fixation times (Fig. 2). There were no significant differences among the 7 groups: 0.5% 1 min vs. 0.5% 10 min, 1% 1 min vs. 1% 5 min, 1% 1 min vs. 5% 5 min, 0.5% 5 min vs. 1% 10 min, 1% 10 min vs. 5% 10 min, 1% 10 min vs. 5% 30 min, or 5% 10 min vs. 5% 30 min. This suggested that the fixation of PMNs with a high concentration of GA inhibited the phagocytosis and produced ROS.

Electron microscopic H_2O_2 demonstration in fixed PMNs

Electron microscopic results showed that after 1 min of mixing, some PMNs attached to particles and exhibited mild deposits in their secretory vesicles (Fig. 3). When PMNs engulfed particles, free radical-producing vesicles had enhanced reaction deposits 10 min later and fused to the phagosomal membrane, releasing vigorous free radicals into the lumen (Fig. 4). Time-dependent H_2O_2 production was enhanced in the secretory vesicles, some of which were fused exactly to phagosome membranes.

Discussion

PMNs were mixed with OPZ and incubated in a cerium medium containing $CeCl_3$ and glucose in physiological saline before fixation (Ohno *et al.*, 1982; Hirai *et al.*, 1991). The glucose is the source for NADPH through the monophosphate shunt in living PMNs. Produced H_2O_2 is captured by cerium ions to form cerium perhydroxide deposits. To electron microscopically observe

such dynamic processes through enzyme translocation from the vesicles to phagosomes, each step should be stopped without any damage to enzyme reactivity. For this purpose we proposed that pure GA could preserve several unstable enzyme activities (Hirai et al., 1989). Kobayashi and Seguchi (1994) and Kobayashi et al., (1999) showed that NADPH oxidase-mediated H_2O_2 production can occur in briefly fixed phorbol myristate acetate-activated granulocytosis (Hirai 1996). In the present study, PMNs were mixed with zymosan particles immediately after GA fixation. These prefixed cells were then incubated in DCFDA medium or cerium medium containing 3 mM NADPH, which cannot be endogenously supplied in fixed cells. The largest fluorescence value occurred with 0.5% GA at 30 min, but we were afraid of contamination or artifacts from that long of a fixation (Fig. 2). Subsequently, electron microscopic results showed that after 1 min of mixing some cells attached to particles and exhibited mild deposits in the secretory vesicles.

When cells engulfed particles, free radical-producing vesicles had enhanced reaction deposits 10 min later and fused to the phagosomal membranes, releasing numerous free radicals into the lumen. Moreover, the Ce reaction deposits were always restricted to the cell-particle interface and to the inner surface of phagosomes facing the ingested particles. No vesicular reaction was observed, because in the phagocytosing living cells, the vesicular fusion to the phagosomal membrane had already advanced during the reaction incubation, and therefore, fixed samples likely showed only the final events.

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