Membrane Fluidity Changes Accompanying Phagocytosis in Normal and in Chronic Granulomatous Disease Polymorphonuclear Leukocytes

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We have studied membrane fluidity changes in polymorphonuclear leukocytes (PMN) during phagocytosis. Membrane fluidity was assessed by electron spin resonance (ESR) using a nitroxide-substituted stearic acid analog (5DS) as a spin probe. PMN from normal subjects and from 3 CGD patients (2 males, 1 female) were incubated in Kreb’s Ringers phosphate with or without opsonized zymosan. ESR spectra were obtained and the order parameter (S), which is inversely related to membrane fluidity, was calculated. Without zymosan addition, S for normal (0.638) and for CGD (0.635) were not significantly different (p > 0.35). The S values indicate that under resting conditions the molecular environment of the CGD membrane is similar to that of normal PMN membranes. However, with addition of opsonized zymosan, the normal, but not the CGD, PMN showed a significant increase (CGD, S = 0.638; normal, S = 0.647; p < 0.001). This change in S for the normals is consistent with a more restricted movement of 5DS. Treatment of normal PMN with a mixture of scavengers specific for H₂O₂ (catalase, 1600 U/ml), O₂⁻ (superoxide dismutase, 100 µg/ml), and for HO⁻ (sodium benzoate, 1mM) during zymosan stimulation gave S values similar to those of resting cells. Catalase alone also lowered S value, suggesting that H₂O₂ was instrumental in causing the initial S value increase. This idea was supported by studies in which CGD cells were incubated with zymosan in the presence of glucose oxidase, an enzyme that catalyzes glucose oxidation resulting in the direct reduction of molecular oxygen to H₂O₂. Our results indicate that reduced O₂⁻ by-products, particularly H₂O₂, can cause altered biophysical properties of PMN membrane during phagocytosis.

S TIMULATED polymorphonuclear leukocytes (PMN) undergo an oxidative burst that generates reduced oxygen by-products including hydrogen peroxide (H₂O₂) and superoxide anion (O₂⁻). The process is beneficial to the host since these highly reactive species participate in the bactericidal mechanisms of the phagocyte. At the same time, however, the oxidative by-products may have detrimental effects on the PMN. For example, the rate of phagocytosis is enhanced in cells incubated under anaerobic conditions as compared to those under aerobic conditions, and cells from donors receiving daily doses of ascorbic acid showed enhanced phagocytosis but released significantly less H₂O₂. Further, H₂O₂ in the presence of halide and myeloperoxidase, a constituent of PMN azurophilic granules, has cytotoxic effects on many different mammalian cells. These findings suggest that H₂O₂ and perhaps O₂⁻ or other oxidative by-products could damage essential cell structures not only in target cells but also in the phagocytes themselves.

A likely target of oxidative attack is the cell membrane. Unsaturated fatty acids are particularly susceptible, and they may participate in a sequence of lipid peroxidative events that elaborate a variety of free radicals. Cross-linking of membrane proteins may also occur following their exposure to oxidants. These effects would be expected to influence the biophysical properties of the membrane.

An ascorbate-mediated oxidative attack on membranes produces decreased membrane fluidity. Recently we showed a similar decrease of membrane fluidity in ascorbate-treated PMN from subjects suffering from Chediak-Higashi syndrome (CHS). Our present work is a study of membrane fluidity changes that accompany production of oxidative by-products in normal PMN during phagocytosis. Since the act of ingestion itself may bring about membrane fluidity changes unrelated to oxidative attack, we have also studied cells of chronic granulomatous disease (CGD) patients. The CGD cells ingest particles in a normal fashion but fail to undergo the oxidative burst and do not generate reduced oxygen by-products. Hence, the CGD PMN should serve as useful controls in distinguishing fluidity effects of oxidative by-products from changes occurring because of membrane perturbation related to internalization of particles.

MATERIALS AND METHODS

Stimulating Agents

Latex (Difco Laboratories, Detroit, Mich.) was exhaustedly diazylated against 0.9% NaCl, suspended in 0.9% NaCl at a concentration of 15 mg/ml, and stored frozen (~70°C). Zymosan (Sigma Chemical Co., St. Louis, Mo.) was boiled in 0.9% NaCl, cooled,
washed once in 0.9\% NaCl, and then suspended at 66 mg/ml in 0.9\% saline. One milliliter samples were stored as described for latex.

**Enzymes**

Glucose oxidase, catalase, and superoxide dismutase (SOD) were obtained from Sigma Chemical Company. Stock solutions in 0.9\% NaCl were prepared shortly before use.

**Cell Preparation**

Human leukocytes (98\%-99\% PMN) were prepared from heparinized venous blood by dextran sedimentation and Ficoll-Hypaque gradient.\(^{15}\) Cells were suspended in phosphate-buffered saline containing 100 mg/dl glucose (PBS.G) at a concentration of 3.5-4.2 \times 10^7 cells/ml.

**Incubation**

A 1-ml sample of PMN in PBS.G was centrifuged (150 g, 2.5 min, 25\°C) in a plastic snap-cap tube (12 \times 75 mm) and the pellet was suspended in 0.9 ml Krebs Ringers phosphate with 100 mg/dl glucose (KRP.G). Addition of particles and/or other agents to be tested were made to give a total volume of 1.0-1.2 ml. The tube was incubated at 37\°C with gentle tumbling (12 rpm) for 15 min.

**Spin Labeling**

The lipid-specific spin label 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl (5DS) was purchased from Syva (Palo Alto, Calif.). A stock solution of 5 mM was made up in ethanol. After the above incubation, the cells or cell-particle suspensions were transferred to a glass tube (13 \times 100 mm) in which 8 \mu l of 5DS had previously been dried to a thin film under a N2 stream. The tube was incubated at 25\°C for 5 min to allow the spin label to diffuse into the cell membrane. The suspension was then centrifuged (40 sec, 400 g, 25\°C) and varying amounts of supernatant removed (see below). After suspension in the remaining volume, the sample was placed in a 100 \mu l capillary and the electron spin resonance (ESR) spectrum obtained. Alternatively, some spin-labeled suspensions were further centrifuged into the heat-sealed tip of the capillary to attain a cell pellet before the spectrum was recorded.

The changes in membrane fluidity observed after stimulation with particles (see Figs. 2 and 3) were independent of the final cell density, which was varied from approximately 4 \times 10^7 PMN/ml to over 10^9/ml. The majority of the ESR measurements were carried out at cell densities greater than 4 \times 10^7 PMN/ml, since the signal-to-noise ratio of the ESR spectra was optimal at that density. Viability, as assessed by trypan blue exclusion after the above protocol, was greater than 99\% and 90\% for the cell and cell-particle suspensions, respectively, when the final cell density was 4 \times 10^8 PMN/ml.

**ESR Data Acquisition and Analysis**

ESR spectra were obtained on a standard balanced-bridge spectrometer with diode detection operating at 9.1 GHz. First derivative absorption spectra were recorded with a 100 gauss (G) field sweep, a scan time of 10 min, a peak-to-peak modulation amplitude of 1.5 G, and a time constant of 0.5 sec. Other instrument settings and data analyses were as previously described.\(^{12}\) In brief, the order parameter (S), a measurement of spin label order and motion, was calculated\(^{16}\) from the extrema of the ESR spectrum of 5DS-labeled PMN (Fig. 1):

\[ S = \frac{T_{11} - T_{1'} - C}{T_{11} + 2T_{1'} + 2C} \cdot 1.66 \]

where C = 1.4 - 0.053 (T_{11} - T_{1'}). As flexibility of the hydrocarbon chains of the spin label decreases, the order parameter increases. This implies that as the environment surrounding the nitroxide group of the label becomes more ordered (i.e., less fluid), the order parameter increases. The theoretical limits of S are S = 0 for a completely fluid, isotropic system, and S = 1 for a completely rigid or ordered environment. Typical values for spin-labeled membranes are from 0.5 to 0.7. Small changes in S may be biologically

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**Fig. 1.** First derivative ESR spectrum of 5DS-labeled normal human PMN taken at 25\°C. The hyperfine splitting parameters 2T_{11} and 2T_{1'} are the separation between outer and inner extrema, respectively, and are used in the calculation of the order parameter. Incubation and labeling conditions and instrument settings are described in Materials and Methods.
Normal CGD Normal CGD
7 7 4 4

RESULTS

The order parameter, S, is a measure of membrane fluidity, and reflects the degree of freedom of molecular motion within the membrane. The relationship of S and fluidity is an inverse one, with higher order parameters indicating less fluid environments for the

\[
P \text{ value} \\
> 0.60 \\
< 0.04 \\
> 0.60 \\
< 0.01
\]

For example, a 10% change in the number of unsaturated fatty acids is reflected in a change in the order parameter of 1%. For experiments in which identical samples were run on the same day, the mean and standard error (SE) were calculated. For experiments in which several samples were measured and data from several days were available, a two-way analysis of variance was carried out. All p values given herein are for the probability that a null hypothesis is true.

![Fig. 2. Order parameters at 25°C for 5DS-labeled normal and CGD PMN before or after stimulation with latex beads (3.75 mg/ml). PMN in 0.9 ml KRP.G were incubated in a snap-cap plastic tube, 13 x 75 mm, with 300 μl latex suspension (4.5 mg latex) for 15 min at 37°C with slow tumbling (12 rpm). The cell-particle suspension was then transferred to a tube containing spin label. Spin labeling and ESR measurements were performed as described in Materials and Methods. Bars indicated 2 SE above the mean of the three experiments. p values are for the probability that a null hypothesis comparing the indicated values is true.](image)

![Fig. 3. Order parameters at 25°C for 5DS-labeled normal and CGD PMN before or after stimulation with opsonized zymosan. PMN in 1 ml of KRP.G were incubated with the opsonized zymosan (5.0 mg/ml) for 15 min at 37°C with slow tumbling (12 rpm). Spin labeling and ESR measurements were performed as described in Materials and Methods. Bars indicate 2 SE above the mean of four experiments. p values are for the probability that a null hypothesis comparing the indicated values is true.](image)
Since catalase removes $\text{H}_2\text{O}_2$ from reaction mixture, we concluded that $\text{H}_2\text{O}_2$ might be altering the cell membrane to bring about a decreased fluidity. We tested this idea by incubating CGD cells with glucose oxidase, an enzyme that catalyzes a reaction giving rise to $\text{H}_2\text{O}_2$ and gluconic acid. Figure 5 shows that glucose oxidase does bring about a significant increase in $S$ ($p < 0.01$) for CGD cells incubated with opsonized zymosan.

DISCUSSION

The application of ESR to measuring membrane fluidity of biologic membranes requires a spin label, a molecule with an unpaired electron ‘‘reporter’’ group such as nitroxide. In our present study we have employed 5DS, an analogue of stearic acid, with the nitroxide group located at the fifth carbon of the hydrophobic chain. 5DS orients in the membrane such that the carboxyl group is at the surface associated with the polar phospholipid head groups, while the hydrocarbon chain aligns with the chains of the acyl groups of the phospholipids. This configuration places the reporter group near the membrane surface but within the hydrophobic portion of the bilayer. Initially the probe enters the external layer of the plasma membrane bilayer and from there will diffuse to the inner layer. Since phagocytosing cells will have invagination of plasma membranes as well as fusion of plasma membrane and granules, it is likely that 5DS also gains access to internal cell membranes. Thus, the spin label technique is one that presents an average membrane fluidity for all the regions probed.
The unpaired electron in the nitroxide group of 5DS is sensitive to a variety of environmental influences, including the order inherent in the bilayer structure and the molecular motion of the spin label. Under our experimental conditions, the two most likely types of molecular motion the reporter group will experience are rotation of the hydrocarbon chain about its own axis and a wobble of this axis relative to the plane of the bilayer. With these considerations in mind, we arrive at a definition of fluidity for purposes of our discussion: the relative freedom of rotational movement experienced by 5DS at its intramembrane location in PMN. We use as a quantitative measure the order parameter, S, which has an inverse relationship to fluidity.

S values for resting PMN from both normal and CGD subjects are similar, indicating that the region of the membrane lipid bilayer probed by 5DS has the same fluidity in the two cell types. However, upon stimulation with a phagocytizable particle, normal, but not CGD, PMN show a marked increase in order parameter. This change in S can be interpreted as indicating a decrease in 5DS motion and, therefore, a decrease in membrane fluidity.

Two lines of evidence suggest that the generation of oxidative by-products, particularly of H$_2$O$_2$, is important in the loss of PMN membrane fluidity. First, incubation of normal phagocytosing PMN with catalase results in a S value nearer to that found in resting cells. Second, incubation of CGD phagocytosing PMN cells with glucose oxidase, an enzyme that catalyzes the direct reduction of oxygen to H$_2$O$_2$, results in an increased S value. In an in vitro system, we have previously suggested that H$_2$O$_2$ is the likely agent that increases S in ascorbate-treated PMN from subjects suffering from Chediak-Higashi syndrome.

Other workers have shown that peroxidative attack, possibly mediated through H$_2$O$_2$, results in increased S values in other membranes. Although H$_2$O$_2$ is important in decreasing membrane fluidity of phagocytosing PMN, other possible sources of S value changes should be considered. Uptake of spin label into zymosan (which results in a highly rigid ESR spectrum) could cause an increase of S value unrelated to PMN fluidity changes. This effect is probably not significant in our spectral measurements, however, since the CGD cells fail to show a significant order parameter increase when they were incubated with zymosan. Another possible source of fluidity change, unrelated to the evolution of H$_2$O$_2$, is the physical change occurring in the phagocyte membrane because of the act of ingestion. The portions of membrane internalized during phagocytosis are not randomly assigned. Furthermore, studies where the fluidity of postphagocytic plasma membranes was assessed by means of fluorescence depolarization revealed that the membrane had altered microviscosity. The contribution of this rearrangement of membrane to the fluidity changes herein described is probably not significant. Again, as with the question of zymosan uptake of spin label, the CGD cells serve as useful controls, and since these latter PMN fail to show the marked increase in
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S, we can conclude that SDS does not report on this phenomenon.

We were able to abolish part of the S increase in normal phagocytosing cells with catalase. However, even at levels of catalase that should completely eliminate the H2O2 being released from the cells, we do not find a complete restoration of resting membrane fluidity. While this might be due, in part, to H2O2 evolution within the phagolysosome, where ingested catalase would not function optimally because of low pH, we cannot rule out the possibility that there are other factors contributing to fluidity decrease.

REFERENCES

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