An Oxygen-Dependent Mechanism of Neutrophil-Mediated Cytotoxicity

By Stephen J. Weiss and Albert F. LoBuglio

Human neutrophils stimulated with phorbol myristate acetate were able to rapidly destroy autologous red blood cell targets. Neutrophil-mediated cytotoxicity was related to phorbol myristate acetate concentration and neutrophil number. The ability of stimulated neutrophils to lyse red blood cell targets was markedly impaired by catalase or superoxide dismutase but not by heat-inactivated enzymes or albumin. Despite a simultaneous requirement for $O_2^-$ and $H_2O_2$ in the cytotoxic event, a variety of $OH^-$ and $O_2$ did not effect cytolysis. The myeloperoxidase inhibitor cyanide did not reduce red blood destruction, while azide consistently impaired cytolysis. The inability of cyanide to reduce cytotoxicity coupled with the protective effect of superoxide dismutase suggests that cytotoxicity is independent of the classic myeloperoxidase-$H_2O_2$-halide system. We propose that neutrophils, stimulated with phorbol myristate acetate, generate $O_2^-$ and $H_2O_2$, which play an integral role in a novel cytotoxic mechanism.

Blood polymorphonuclear leukocytes (PMNs) play an integral role in host defense and are capable of destroying a wide variety of targets, including bacteria, fungi, red cells, and malignant or nonmalignant nucleated cells. Although the mechanisms of target cell destruction are not well understood, it appears that the utilization of $O_2$ by the PMN plays an important role. Following specific membrane perturbation, the PMN exhibits a burst in $O_2$ consumption (termed the respiratory burst) associated with the generation of superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), and possibly the hydroxyl radical (OH) and singlet oxygen ($O_2$). Cells incapable of a respiratory burst from patients with chronic granulomatous disease have been reported to have impaired ability to destroy antibody-coated bacteria, fungi, and malignant cells. Generation of oxidant species by cell-free model systems appear capable of destroying a variety of target cells. However, little work has dealt with the cytotoxic potential of the oxidizing species generated by the intact PMN. In this study, we demonstrate the rapid destruction of red blood cell (RBC) targets by human PMNs exposed to phorbol myristate acetate (PMA), a potent stimulus of leukocyte oxidative metabolism. PMNs appear to mediate this cytotoxicity by a mechanism dependent on both $O_2^-$ and $H_2O_2$.

MATERIALS AND METHODS

PMN preparations were isolated from the blood of normal volunteers by dextran sedimentation with or without further purification.

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BLOOD polymorphonuclear leukocytes (PMNs) play an integral role in host defense and are capable of destroying a wide variety of targets, including bacteria, fungi, red cells, and malignant or nonmalignant nucleated cells. Although the mechanisms of target cell destruction are not well understood, it appears that the utilization of $O_2$ by the PMN plays an important role. Following specific membrane perturbation, the PMN exhibits a burst in $O_2$ consumption (termed the respiratory burst) associated with the generation of superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), and possibly the hydroxyl radical (OH) and singlet oxygen ($O_2$). Cells incapable of a respiratory burst from patients with chronic granulomatous disease have been reported to have impaired ability to destroy antibody-coated bacteria, fungi, and malignant cells. Generation of oxidant species by cell-free model systems appear capable of destroying a variety of target cells.

RESULTS

As summarized in Table 1, neither PMA nor PMNs alone were capable of mediating significant RBC lysis, while PMA-stimulated PMNs destroyed three-fourths of the RBC targets. Thus, each PMN lysed more than 7 RBC targets under these conditions. The degree of

% cytotoxicity = \[
\frac{(A - B)}{(C)} \times 100
\]

where A is the mean cpm in the supernatant of samples containing PMA-treated PMNs and RBCs, B is the mean cpm in the supernatant of samples containing RBCs alone, and C the mean cpm of RBCs added to each sample. Other additions included bovine superoxide dismutase (SOD, 3000 U/mg, Sigma Chemical Co., St. Louis, Mo.), bovine catalase (type C-40, Sigma Chemical Co.), bovine albumin (fatty-acid-free, Sigma Chemical Co.), sodium azide, sodium cyanide, mannitol (Fisher Scientific Co., Fair Lawn, N.J.), L-histidine, 2-keto-4-thiophenylbutyric acid (Sigma Chemical Co.), and 2.5 dimethylfuran (Aldrich Chemical Co., Milwaukee, Wisc.). The catalase employed was free of contaminating SOD activity and the 2.5 dimethylfuran was purified before use by passage over a column of aluminum oxide (W200 basic, International Chemical and Nuclear Corp.). Identical numbers of target cells were added with or without the above agents in the absence of PMNs to determine the spontaneous release of $^{51}$Cr.
target cell destruction was confirmed with RBC not carrying the $^{51}$Cr label by determining the percent cytotoxicity by direct hemocytometer counts of red cells. In three experiments, 65$\pm$4.2% cytotoxicity was obtained with unlabeled RBC targets. Figure 1A depicts a representative study of PMN-mediated cytotoxicity as a function of PMA concentration. Maximal cytotoxicity could be obtained with doses as low as 1 ng/ml. DMSO alone at the highest concentrations employed produced no cytotoxicity. Figure 1B illustrates the direct relationship of PMN number to cytotoxicity. The time course of $^{51}$Cr release is illustrated in Fig. 2, with maximal release occurring by 4 hr. Microscopic examination of lysis followed a time course identical to that obtained with $^{51}$Cr release.

In order to characterize the possible role of PMN-generated $O_2$ metabolites in the cytotoxic event, experiments were performed in the presence of SOD or catalase. Table 2 lists the results of an experiment designed to determine the role of $O_2^-$ and/or $H_2O_2$ in target cell destruction. SOD or catalase were both capable of almost completely inhibiting cytolysis. Heat-inactivated enzymes or albumin at concentrations up to 300 $\mu$g/ml did not inhibit RBC destruction. In 10 experiments, SOD and catalase inhibited RBC lysis by 83$\pm$7% and 94$\pm$5%, respectively. These data indicate a clear role for both $O_2^-$ and $H_2O_2$ in the lytic event. The addition of SOD or catalase 60 min after the start of the incubation had no inhibitory effect on cytolysis. Thus, it appears that $O_2^-$ and $H_2O_2$ mediate maximal target cell damage during the first hour of incubation.

The extremely short half-life of reactive oxygen metabolites should place restrictions on their ability to diffuse from the PMN to the RBC target. Resuspension of pelleted PMNs and RBCs every 10 min during the first hour of incubation completely inhibits cytolysis over the 4-hr period. Control samples that were resuspended and then repelleted every 10 min during the first hour of incubation destroyed over 70% of the targets. It appears that intimate cell–cell contact is required for maximal cytolysis.

$O_2^-$ and $H_2O_2$ in the presence of suitable catalysts may interact to form extremely powerful oxidants, the $OH^-$ and/or $^{1}O_2$. If the $OH^-$ or $^{1}O_2$ are involved in cytolysis, then compounds known to scavenge these species should inhibit RBC destruction. We
examined a wide range of concentrations of these inhibitors up to maximal doses of 40 mM ethanol or mannitol, 2 mM 2-keto-4-thiromethylbutyric acid, 20 mM histidine, and 1 mM 2,5 dimethylfuran without evidence of inhibition (Table 3). In addition, the effect of the scavengers on the rate of cytolysis was determined by measuring ⁵¹Cr release after 1, 1.5, 2, 2.5, and 3 hr of incubation. None of the scavengers, at the highest concentrations studied, had any effect on the rate of RBC lysis. In an attempt to accentuate competition for the oxygen metabolites, we also examined the effects of these scavengers at maximal dosage with decreasing numbers of effector cells without evidence of inhibition (data not shown).*

In order to rule out the formation of a stable lytic species dependent on O₂⁻ and H₂O₂ for its generation, reaction mixtures of PMA-stimulated PMNs with or without unlabeled RBCs were incubated for 2 hr. At the end of this period, ⁵¹Cr-labeled RBC targets were added to the mixtures and incubated for an additional 4 hr. Neither of the reaction mixtures were capable of mediating ⁵¹Cr release from the labeled RBCs during the 4-hr incubation.

The myeloperoxidase-H₂O₂-halide system has been demonstrated capable of exerting a powerful microbiocidal and cytotoxic effect. In order to determine the role of this enzyme system in RBC destruction, experiments were performed in the presence of azide or cyanide, potent inhibitors of myeloperoxidase activity. Azide clearly protected RBCs at all concentrations employed, while cyanide had no inhibitory effect on cytolysis at the highest concentrations studied (Table 4). Indeed, lower concentrations of cyanide actually enhanced target cell destruction.

**DISCUSSION**

In this study, human neutrophils stimulated with PMA were capable of mediating RBC cytolysis. Target cell destruction was directly related to the number of PMNs and the PMA concentration employed. Although PMA is not a “physiologic” stimulus, this compound causes a marked stimulation of all parameters of leukocyte oxidative metabolism normally associated with phagocytosis. Thus, PMA stimulates PMN hexose monophosphate shunt activity, chemiluminescence, protein iodination, and the

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**Fig. 2.** The release of ⁵¹Cr from 1.25 x 10⁶ RBC targets incubated with 1.25 x 10⁶ PMNs and 2.5 ng/ml of PMA over a 5-hr period of incubation. The spontaneous release from the RBC targets at 5 hr was 1.9%. Results are expressed as the mean ± 1 SD of triplicate samples in a single representative experiment of 5 performed.

**Table 2.** Effect of SOD and Catalase on PMN-Mediated Cytotoxicity

<table>
<thead>
<tr>
<th>Additive*</th>
<th>Percent Cytotoxicity †</th>
<th>Percent Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>85.1 ± 1.1</td>
<td>—</td>
</tr>
<tr>
<td>SOD (15 μg/ml)</td>
<td>14.3 ± 0.7</td>
<td>83.2</td>
</tr>
<tr>
<td>Catalase (75 μg/ml)</td>
<td>3.1 ± 0.4</td>
<td>96.4</td>
</tr>
<tr>
<td>Albumin (300 μg/ml)</td>
<td>86.4 ± 1.2</td>
<td>—</td>
</tr>
<tr>
<td>Heat-inactivated SOD (15 μg/ml)</td>
<td>79.1 ± 0.8</td>
<td>7.0</td>
</tr>
<tr>
<td>Heat-inactivated catalase (75 μg/ml)</td>
<td>83.4 ± 0.6</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*Each reaction mixture contained 1.25 x 10⁶ PMN, 1.25 x 10⁶ RBC, and 2.5 ng/ml PMA. The duration of incubation was 4 hr.
†Results expressed as the mean ± 1 SD of triplicate samples in a single representative experiment of 10 performed.
‡Enzymes were heat inactivated in a boiling water bath.

**Table 3.** Effect of OH⁻ and O₂ Scavengers on Neutrophil-Mediated Cytotoxicity

<table>
<thead>
<tr>
<th>Additive*</th>
<th>Percent Cytotoxicity †</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>75.4 ± 15.0</td>
</tr>
<tr>
<td>Ethanol (40 mM)</td>
<td>74.4 ± 11.9</td>
</tr>
<tr>
<td>Mannitol (40 mM)</td>
<td>67.5 ± 14.9</td>
</tr>
<tr>
<td>2-Keto-4-thiomethylbutyric acid (2 mM)</td>
<td>72.4 ± 15.5</td>
</tr>
<tr>
<td>2,5 Dimethylfuran (1 mM)</td>
<td>76.2 ± 14.3</td>
</tr>
<tr>
<td>Histidine (20 mM)</td>
<td>72.3 ± 14.4</td>
</tr>
</tbody>
</table>

*Each reaction mixture contained 1.25 x 10⁶ PMN and 1.25 x 10⁶ RBC. Drugs were added to the mixtures 5 min before the addition of 2.5 ng/ml PMA. None of the compounds tested increased ⁵¹Cr release alone. The duration of incubation was 4 hr.
†Results expressed as mean ± 1 SD of 6 experiments.

*In initial experiments, solutions of scavengers were prepared in commercial siliconized tubes (Becton-Dickenson and Co., Rutherford, N.J.). Compounds prepared in these tubes inhibited RBC destruction artifically due to the release of inhibitory compounds from the siliconized glass. Scavengers were subsequently prepared in polypropylene tubes.
The destruction of RBC targets by PMA-stimulated PMNs was almost completely inhibited by SOD or catalase, indicating that neither $\text{O}_2^\cdot$ nor $\text{H}_2\text{O}_2$ were individually capable of target cell destruction. The cytolyis required both $\text{O}_2^\cdot$ and $\text{H}_2\text{O}_2$, but could not be inhibited by a variety of compounds known to be $\text{OH}^\cdot$ or $\text{O}_2^\cdot$ scavengers. Interestingly, Kellogg and Fridovich have demonstrated that oxygen metabolites generated by the acetaldehyde-xanthine oxidase system rapidly destroyed human RBCs in a glucose-free medium. In their study, cytolyis was inhibited by SOD or catalase, but mannitol, histidine, and 2,5 dimethylfuran were also protective. In this study, these agents, even at higher concentrations, failed to inhibit cytolyis. At present, we are unable to explain these differences, although several explanations are possible. First, the lytic agent(s) generated by the acetaldehyde-xanthine oxidase system may differ from the PMN-derived species. Acetaldehyde may not only act as a substrate for xanthine oxidase but also interact with the oxygen metabolites generated to form other reactive species. Second, the differences in the rate of oxygen metabolite generation by the acetaldehyde-xanthine oxidase system and the PMN may play an important role in the mechanism of cytolyis. In early experiments studying the RBC defense mechanisms against oxidant stress, Cohen and Hochstein demonstrated that the response of the RBC to $\text{H}_2\text{O}_2$ was dependent on its concentration and rate of generation. Third, target cell lysis in our system may require interaction of both $\text{O}_2^\cdot$ and $\text{H}_2\text{O}_2$ directly with the RBC membrane without a requirement for the formation of a third species. $\text{O}_2^\cdot$ and $\text{H}_2\text{O}_2$ may sequentially interact with polyunsaturated fatty acids in the target cell membrane to initiate and propagate the formation of lipid peroxides capable of disrupting cell architecture. Fourth, we cannot rule out the possibility that the scavengers themselves may form potentially cytotoxic oxidants after reacting with neutrophil-derived oxygen metabolites. Finally, the scavengers employed may not be capable of competing adequately for $\text{OH}^\cdot$ and/or $\text{O}_2^\cdot$ interaction with the target cell surface. Although the lack of accessibility of the scavengers at the site of oxygen metabolite generation might explain their lack of protective action; the ability of the larger molecular species, i.e., SOD and catalase, to inhibit cytolyis makes this explanation less likely.

In contrast to the free radical theory of hemolysis, Rodgers et al. recently proposed that a stable toxic material can be obtained from peroxidized microsomal lipids that is capable of mediating hemolysis. In this study, we could not demonstrate the formation of a stable species by stimulated PMNs capable of mediating RBC destruction.

The myeloperoxidase-$\text{H}_2\text{O}_2$-halide system described by Klebanoff is capable of destroying a variety of targets, including bacteria, fungi, viruses, and tumor cells (for review see ref. 2). In one of the few studies examining the mechanism of cell-mediated cytotoxicity by the intact PMN, Clark and Klebanoff examined the interaction of human PMN (stimulated with opsonized zymosan) with a murine tumor cell (LSTRA) at an effector:target cell ratio of 2.5:1. Although the degree of cytolysis was modest (a doubling of spontaneous release), strong evidence was presented for the role of the myeloperoxidase-$\text{H}_2\text{O}_2$-halide mechanism of target cell damage. The effects of SOD or $\text{OH}^\cdot$ scavengers were not reported. The cell-free myeloperoxidase system is also capable of destroying human RBC targets. Hemolysis was inhibited by the peroxidase inhibitors azide or cyanide and by catalase, but was stimulated by SOD when xanthine–xanthine oxidase was used as a source of $\text{H}_2\text{O}_2$. In this study, the lack of an inhibitory effect by cyanide on cytolyis coupled with the protective effect of SOD suggests that the mechanism of cytolyis is independent of the classic myeloperoxidase system. The inhibitory effect of azide is unexplained but similar results are obtained with studies of the human monocyte.

Recently, Nathan et al. examined the interaction of murine peritoneal exudate granulocytes and BCG, C. parvum, or casein activated macrophages stimulated by PMA on a variety of murine tumor cells and murine RBCs. These authors concluded that target cell lysis was due to $\text{H}_2\text{O}_2$ without a requirement for $\text{O}_2^\cdot$, myeloperoxidase, or halide. They demonstrated...
inhibition of cytolysis with catalase, while superoxide dismutase, azide, and scavengers of singlet oxygen and OH· had no adverse effect on cytolysis of the target cells. These studies contrast with our observations and suggest that a variety of factors, including the animal species, source and type of leukocytes, stimulus to induce respiratory burst, and type of target cell, may all influence the oxygen-dependent mechanisms operative in these models of leukocyte-mediated target cell lysis.

In conclusion, this study has examined the cytotoxic potential of leukocyte-generated oxygen metabolites toward RBC targets. Although the identity of the final mediator of target cell destruction is unknown, it apparently requires both O2− and H2O2 for its generation. This cytotoxic system provides novel insight into potential mechanisms of leukocyte-mediated target cell injury.

REFERENCES

An oxygen-dependent mechanism of neutrophil-mediated cytotoxicity

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