Role of Oxygen Radicals in Experimental Allergic Uveitis

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The experimental autoimmune disease elicited by a large dose of retinal S antigen in guinea pigs is characterized by massive necrotizing uveitis and retinitis. Treatment of these animals with the antioxidants superoxide dismutase, catalase, and sodium benzoate resulted in marked reduction of uveal inflammation. The attenuated inflammation was characterized by a relatively well-preserved retina and retinal pigment epithelium, along with a reduction of subretinal exudate and vitreous inflammation. These findings suggest that reactive oxygen metabolites may play a role in the destruction of ocular tissue and amplification of the inflammatory process in experimental uveitis. Invest Ophthalmol Vis Sci 28:886-892, 1987

The damaging effects of inflammation are primarily mediated through chemical substances released by the infiltrating polymorphonuclear leukocytes (PMNs) or monocytes. These chemical mediators include neutral proteases, elastases, cathepsin G, arachidonic acid metabolites, monokines and others.1 Recent studies of the mechanisms of phagocyte-mediated destruction of microbes have revealed a significant increase in oxygen consumption by these inflammatory cells that is associated with the generation of reactive oxygen products. The role of these reactive oxygen metabolites in antimicrobial defense mechanisms has been the subject of intensive investigation. Recently, attention has been focused on the potential significance of these metabolites in the inflammatory destruction of cellular components.2,3

The primary oxygen metabolites include univalent and divalent products of oxygen, superoxide, and hydrogen peroxide.2,4,5 The superoxide is the principal product of the respiratory burst of stimulated neutrophils and mononuclear phagocytes.6 Many of the cytotoxic effects of the superoxide are believed to result from the formation of more reactive species that include hydrogen peroxide and hydroxyl radicals.2,7-11

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Submitted for publication: July 24, 1985.
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The authors previously reported considerable differences in the experimental autoimmune disease elicited by retinal S antigen.12 A massive necrotizing intraocular inflammation was produced by an inoculum of 50 μg of S antigen, although a mild granulomatous panuveitis was induced by injecting 5-10 μg of the antigen. In this article, the authors will report the modulating effects of superoxide dismutase (SOD), catalase and benzoic acid on severe S antigen-induced ocular inflammation.

Materials and Methods

The investigations described in this study conform to the ARVO Resolution on the Use of Animals in Research.

Thirty-six female Hartley strain adult guinea pigs were given a single hind footpad injection of purified bovine soluble retinal antigen in complete Freund’s adjuvant. The authors’ method of isolation and purification of the S antigen was similar to the method described by Dorey et al.13 Each animal received 50 μg of the antigen; all were examined daily for limbal injection, anterior chamber reaction, and vitreous exudate. The guinea pigs were divided into six groups of six animals each and treated as follows. (1) Group 1 animals received daily intraperitoneal (IP) injections of 0.5 ml of normal saline. (2) Group 2 animals received daily IP injections of 2,000 units of polyanethylene-glycol-modified superoxide dismutase (PEG-SOD) (Enzon, Inc.; Piscataway, NJ). (3) Group 3 animals received similar daily IP injections of heat-inactivated polyanethylene-glycol-modified SOD, (PEG-SOD, 2,000 units). (4) Group 4 animals received IP injections of polyanethylene glycol-modified catalase, (PEG-catalase),
2,400 units/kg (Enzon, Inc.; Piscataway, NJ). (5) Group 5 animals received daily IP injections of heat-inactivated polyethylene glycol-modified catalase (PEG-catalase, 2,400 units/kg). (6) Group 6 animals received daily IP injections of sodium benzoate, 100 mg/kg (Aldrich Chemical Co.; Milwaukee, WI).

The IP administrations were begun on the tenth day after the injection of S antigen, and all animals were killed on day 21. The eyes were enucleated, fixed in formalin, then processed for paraffin embedding and hematoxylin–eosin (H–E) staining.

The eyes were coded to ensure that the pathologist was unaware of the treatment at the time of evaluation. One representative slide of each eye from every animal was selected for morphometric analysis. The slide was then magnified ×100 and the outline of the choroid was traced using a camera lucida system.

The choroidal tracing was then analyzed for total choroidal length (perimeter) and total choroidal area, using a Videoplan computer analyzer (Carl Zeiss Inc; West Germany). The average choroidal thickness ("h") of each eye was calculated by dividing the total choroidal length into the total choroidal area. This "h" value correlated (correlation coefficient = 0.9986), with 99.9% confidence limits, to a mean value that was obtained by measuring the thickness of the choroid at 80–100 locations along its entire length. On completion of the morphometry, the code was broken. Mean "h" values of the choroids in control animals, as well as those treated with active and inactive SOD, catalase and sodium benzoate, were then compared using Student's t-test.

**Determination of SOD in Ocular Tissue**

To determine the levels of SOD in ocular tissue following IP injection of this enzyme a group of six normal Hartley strain guinea pigs received IP injections of PEG-SOD (2,000 units); a second group of six received similar IP injections of the same dose of heat-inactivated PEG-SOD; and a third group of six received IP injections of normal saline. Twenty-four hours following this procedure, the animals were killed. The freshly enucleated globes were analyzed for SOD activity essentially as described by Crapo et al.14

Briefly each eye was homogenized in 2 ml of detergent (0.625 mg cholic acid in 100 ml phosphate buffer with 0.1 M EDTA). Tubes were then centrifuged and the supernatant was collected. To 100 μl undialyzed supernatant in a 3-ml cuvette with a light path of 1.0 cm at 24°C, 0.3 ml of 0.1 mM Ferricytochrome C, 0.3 ml of 0.5 mM xanthine and 2.3 ml buffer were added and mixed. The reaction was initiated with 20 μl xanthine oxidase (diluted 1:50 from a stock solution). The rate of increase in absorbance per minute was recorded at 550 nm. In another reaction mixture, the sample was replaced with an equal volume of buffer and the rate recorded after xanthine oxidase was added. The contribution of nonspecific activity to the reduction rate also was taken into consideration. The supernatant first was boiled and then processed as described. The change in absorbance was plotted against SOD concentration. SOD concentration was calculated in units based on the suppressed reduction of Ferricytochrome C in the reaction mixture. One unit is defined as the quantity of SOD required to inhibit the reduction rate of cytochrome C by 50% under the specific condition.14

Protein concentration was determined by the standard Biuret method. Samples obtained from the 18 animals mentioned above were divided into three groups according to the treatments administered. The mean of each group was compared and analyzed using Student's t-test.

**Determination of Catalase in Ocular Tissue**

A group of six Hartley strain guinea pigs received IP injection of 2,400 units of PEG-catalase; a second group of six animals similarly received heat-inactivated PEG-catalase (2,400 units); and a third group in this study received IP injection of normal saline. Twenty-four hours later, the animals were killed and the globes were enucleated. Catalase activity was determined by the method of Aebi.15

Each eye was homogenized and sonicated in 2 ml of 50 mM phosphate buffer (pH, 7.0) containing 0.125 gm cholic acid/50 ml buffer. Tubes were then centrifuged and the supernatant collected. One hundred microliters of supernatant was diluted in 1.9 ml of 50 mM phosphate buffer (pH, 7.0). The solution containing the enzyme and H₂O₂ was read against a control blank solution containing the enzyme and phosphate buffer. To start the reaction, 1 ml of 30 mM H₂O₂ was added. The estimation of catalase activity was based on measurements of a first order rate constant (k) as recommended by Aebi.15 This is derived by measuring the decrease in absorbance over a set time interval. The authors found the decrease in absorbance at ΔA240 nM to be linear for at least 1 min and thus catalase activities were measured over a 1-min interval after initiating the reaction with H₂O₂. The following relationship was used for our calculations:

\[ k = (2.3/60) (\log A_0/A_1) \]

The results are expressed on the basis of protein content in the samples. The protein content of each eye was determined by the Biuret assay method. The mean of each group was compared and analyzed using Student's t-test.
Results

Clinically, all of the experimental and control animals developed signs of uveitis on days 12 or 13 after injection of the S antigen. Histopathologically, the eyes from the control animals (given normal saline, heat-inactivated SOD and heat-inactivated PEG-catalase injections) showed massive infiltration of mononuclear cells, neutrophils, and eosinophils, mainly in the uveal tract but with an extension of cells into the retina and vitreous. Diffuse infiltration was evident throughout the entire uveal tract. The retina at the site of inflammatory cell infiltration was necrotic; the retinal pigment epithelium was both necrotic and disrupted (Fig. 1). In the vitreous, most of the inflammatory cells were found near the pars plana (Fig. 2).

In contrast to these changes, the globes of animals treated with SOD, catalase, and sodium benzoate revealed less inflammatory cell infiltration, with the infiltrate restricted predominantly to the uveal tract (Fig. 3). Although there were occasional foci of inflammation involving the retina, the extent of retinal necrosis and inflammatory cell infiltration was minimal. The retinal pigmented epithelium was relatively uninvolved in the inflammatory process, except at sites where the inflammatory cells extended into the retina. Moreover, in the vitreous, inflammatory cellular exudate was minimal near the pars plana (Fig. 4).

Morphometric analysis of the inflammatory process in the six groups of animals revealed an average choroidal thickness of 194 ± 83 μm in the control (normal saline-treated) group; 124 ± 12 and 188 ± 92 μm in the heat-inactivated SOD-treated and heat-inactivated catalase group, respectively; 60 ± 33 μm in the SOD-treated animals; and 93 ± 93 μm in guinea pigs treated with...
sodium benzoate. In comparison with the control animals, the difference in choroidal thickness in three of the treated groups (SOD, catalase, and sodium benzoate) was uniformly significant at $P < 0.001$ (Table 1). A similarly significant reduction ($P < 0.001$) in choroidal thickness was noted when the SOD-treated group was compared with the group treated with heat-inactivated SOD. Similar significant reduction was noted when PEG-catalase group was compared with the group treated with heat-inactivated PEG-catalase.

Table 1. Results of morphometric analysis of choroidal thickness in animals treated with antioxidants and hydroxyl radical scavenger

<table>
<thead>
<tr>
<th>Treatment groups*</th>
<th>Choroidal thickness (μm)</th>
<th>Significance†</th>
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<tbody>
<tr>
<td>Normal saline</td>
<td>194 ± 83</td>
<td></td>
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<tr>
<td>Superoxide dismutase</td>
<td>80 ± 7</td>
<td>$P &lt; 0.001$</td>
</tr>
<tr>
<td>Heat-inactivated SOD</td>
<td>124 ± 12</td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
<td>60 ± 33</td>
<td>$P &lt; 0.001$</td>
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<tr>
<td>Heat-inactivated catalase</td>
<td>188 ± 92</td>
<td></td>
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<tr>
<td>Sodium benzoate</td>
<td>93 ± 93</td>
<td>$P &lt; 0.001$</td>
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* Six animals in each group.
† Experimental groups were compared with the normal saline group using Student's t-test.

Superoxide Dismutase in Ocular Tissue

Eyes obtained from the injected animals revealed the following mean values of SOD activity: 2.73 ± 0.26 units/mg of protein (active SOD group); 1.61 ± 0.12 units/mg (heat-inactivated SOD group); and 1.62 ± 0.13 units/mg (normal saline control group). There were significantly higher levels of SOD in the eyes obtained from SOD-injected animals when compared with the heat-inactivated SOD and normal saline groups ($P < 0.001$) (Table 2). Based on the values of boiled homogenates, the authors found that nonspecific activity in no way contributed to the reduction of cytochrome C.

Catalase in Ocular Tissue

Catalase activity in three groups of animals is summarized in Table 3. There was no significant difference...
in the activity of this enzyme in ocular tissue of animals injected with normal saline (k = 2.20 ± 0.07/mg) when compared with the group treated with heat-inactivated PEG-catalase (k = 2.29 ± 0.12/mg). The animals injected with PEG-catalase (k = 3.28 ± 0.24/mg) showed significantly higher levels of catalase activity when compared with the other two groups (P < 0.001).

Discussion

Administration of antioxidant enzymes or a hydroxyl radical scavenger reduced the severity of ocular inflammation and the extent of tissue necrosis in severe experimental allergic uveitis (EAU). Although there is no direct evidence that reactive oxygen metabolites are generated in EAU, the reduction in inflammatory components achieved by the antioxidant enzymes and hydroxyl radical scavenger indicates that reactive oxygen metabolites play a role in the inflammatory tissue destruction evidenced in severe EAU.

It has been demonstrated that superoxide can generate chemotactic factors for neutrophils. Such generation can lead to the recruitment of polymorphonuclear leukocytes, which in turn perpetuates and amplifies the inflammatory response. The reduced severity of ocular inflammation and tissue destruction in the animals treated with SOD may be due to partially reduced recruitment of inflammatory cells.

The leukocytic neutral proteases released during inflammation can inflict damage on the extracellular components; normally such damage is prevented by antiproteases that are present in the tissue. Because superoxide radicals are known to inactivate these antiproteases, it follows that the reduced tissue damage and inflammation observed in animals treated with SOD could also have resulted from the antiprotease protection mechanism.

In the experimental immune complex disorders of skin, lung, and kidney, the inflammatory tissue damage was modulated by catalase; however, these were short-term experiments that lasted less than 24 hr. The authors’ observations indicate that antioxidants may be capable of providing extended protection against inflammation. The severity of S antigen-induced ocular inflammation could be significantly reduced over a week-long period with the use of SOD and catalase. This extended antiphlogistic activity may result from the abundance and strategic distribution of antioxidants in the ocular structures, when compared with distribution in lung and other organs. It is also well established that enzyme antioxidants not only prevent the initial direct tissue damage induced by oxygen metabolites but also reduce the severity of ongoing ocular inflammation by preventing the generation of chemotactic factors and protecting the activity of antiproteases.

Under the conditions of this experiment, catalase proved equal to SOD in its effectiveness as an antiphlogistic agent. The authors reported high levels of catalase activity in ocular tissue of rats with lens-induced uveitis following IP injections of polyethylene-modified catalase. Similarly, current studies of IP injections of catalase and SOD revealed that high levels of this enzyme persist in ocular tissue for up to 24 hr (Tables 2, 3). Most of these enzymic activities could be in the uvea, which lacks a blood-ocular barrier. By determining SOD and catalase levels in various ocular structures following IP injection and dose-response studies in EAU, we may be able to provide additional data on the significance of the blood-ocular barrier and distribution of these antioxidant enzymes in normal and inflamed eyes.

The heat-inactivated SOD treatment reduced choroidal inflammation (Table 1), but not to the degree that was achieved with the active SOD treatment. This finding suggests that the preservatives present in enzymes that function as antioxidants may suppress inflammation to some extent. Additional studies are in progress that will serve to delineate the nature of these preservatives and their role in reducing S antigen-induced uveitis. In contrast to the effects of heat-inacti-

### Table 2. Ocular levels of superoxide dismutase activity after intraperitoneal injections of PEG-superoxide dismutase in guinea pigs

<table>
<thead>
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<th>Experimental groups</th>
<th>units/mg protein* †</th>
<th>P value</th>
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<td>Normal saline injected (control)</td>
<td>1.62 ± 0.13</td>
<td>† Values are the means of six samples ± SEM.</td>
</tr>
<tr>
<td>Heat-inactivated PEG-SOD (inactive SOD)</td>
<td>1.61 ± 0.12</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>PEG-SOD (active SOD)</td>
<td>2.73 ± 0.26</td>
<td>P &lt; 0.001</td>
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### Table 3. Ocular levels of catalase activity after intraperitoneal injections of polyethylene-glycol-modified catalase (PEG-catalase) in guinea pigs

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<td>P &gt; 0.05</td>
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<tr>
<td>PEG-catalase (active catalase)</td>
<td>3.28 ± 0.24</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
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* Values are the means of six samples ± SEM.
† The content of catalase is based on the constant rate of a first-order reaction as described by Arrhenius.
duced SOD on the inflammation, heat-inactivated PEG-catalase had no effect on the uveal inflammation, indicating the specificity of catalase in reducing the severity of experimental uveitis.

Benzoic acid and its salt, sodium benzoate, have been used as antioxidants in a variety of situations. Benzoic acid is widely and safely used as a food preservative, and as a preservative of numerous topical ocular drugs. The amount of carbon dioxide released from benzoic acid is a common assay for hydroxyl radical production in both chemical and cellular-generating systems. Treatment with sodium benzoate resulted in reduced inflammatory cell infiltration in the choroid and retina. Similar to the effects of antioxidant enzymes, this reduction of inflammatory cells was associated with a relatively well-preserved retina and retinal pigment epithelium. Of the various inflammatory cells, polymorphonuclear leukocytes were most markedly diminished in all treatment groups.

In view of the long-term treatments applied in these experiments, the antiphlogistic activities of SOD, catalase, and benzoic acid cannot be entirely attributed to the prevention of direct cytotoxic effects brought on by reactive oxygen metabolites. The authors observed that the numbers of neutrophils were reduced by antioxidant treatment; thus, inflammatory amplification mechanisms were clearly modulated. Presumably, the cytolytic effects of leukocyte proteases also were reduced, possibly through the natural protection of an enzyme. The authors observed that the numbers of neutrophils were reduced by an antioxidant treatment; thus, inflammatory amplification mechanisms were clearly modulated. Presumably, the cytolytic effects of leukocyte proteases also were reduced, possibly through the natural protection of an enzyme.

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Key words: oxygen radicals, free radical scavengers, experimental uveitis, superoxide dismutase, catalase, sodium benzoate, hydroxyl radical

References