A superoxide-producing system in the conjunctival mucus thread. PETER PROCTOR,* DONALD KIRKPATRICK,* and JOHN McGINNESS.**

In normal sterile eyes, the conjunctival mucus thread is capable of reducing the vital stain iodonitrotetrazolium (INT). The greatly increased amount of INT reduction in bacterial conjunctivitis has been used as a clinical test for this disorder. We find that native superoxide dismutase, but not the heat inactivated enzyme, inhibits INT reduction by the conjunctival mucus thread in vitro as well as in vivo in sterile rabbit eyes. Furthermore, 3,4-dihydroxybenzoic acid (DHBA), a scavenger of superoxide radical (O_2^-), inhibits INT reduction by the mucus thread. We conclude that INT reduction by the mucus thread is due to a superoxide radical-producing system similar to that apparently responsible for the tetrazolium-reducing properties of granulocytes and other phagocytes. This oxygen radical-producing system may serve as a defense against infection and possibly as a mediator of the conjunctival inflammatory response.

The tetrazolium dye, iodonitrotetrazolium (INT) is reduced by acellular vacuoles found in the conjunctival mucus thread of human eyes, forming an insoluble violet precipitate. In bacterial conjunctivitis a marked increase in dye reduction is associated with the infiltration of granulocytes into the mucus thread. The biochemical basis for this test was previously undefined.

The reduction by granulocytes of a similar tetrazolium dye, nitroblue tetrazolium (NBT) provides a clinical test for bacterial infection and for distinguishing between normal granulocytes and granulocytes in chronic granulomatous disease. There is evidence (refs. 2 and 3; however, see ref. 4) that this test reflects (at least partially) the production of the bactericidal superoxide anion radical (O_2^-) by the granulocyte: O_2^- + tetrazolium dye → reduced tetrazolium dye (insoluble) + O_2.

The clinical and biochemical similarities between vital staining of the conjunctival mucus thread with INT and the measurement of superoxide production with NBT in granulocytes suggested that INT reduction reflects a superoxide-producing system in the mucus thread and possibly in the conjunctiva. We have confirmed this hypothesis using two separate and complementary tests for superoxide production.

Methods

In vivo studies. A 0.01 ml aliquot of a 5 mg/ml solution of superoxide dismutase (SOD) (Miles Laboratories, Kankakee, Ill., 11,500 u/mg) was instilled into the temporal conjunctival sack of the left eye of four New Zealand white male rabbits (5 to 7 lb.). An aliquot of autoclaved SOD was instilled into the right eye. After 7 min., a 0.01 ml aliquot of a 1.0% solution of INT was instilled into both eyes. Two hours later, the mucus threads were removed from both eyes and mounted on glass slides for microscopic viewing.

In vitro studies. To increase the amount of available conjunctival mucus, a mild sterile conjunctivitis was induced by the instillation of drops of ether into the eyes of New Zealand white male rabbits (5 to 7 lb.) previously sedated with 50 mg of chlorpromazine administered intramuscularly. The mucus threads were harvested as they appeared and divided into approximately equal sections (1 to 2 mm wide) on a glass slide. A 0.005 ml aliquot of a 5 mg/ml solution of either native or heat-inactivated SOD in balanced salt solution was instilled onto a section of mucus thread, which was incubated at 25°C for 5 min. in a closed, moisture-saturated Petri dish. A similar section of the same mucus thread was treated with a 0.005 ml of a 3 mg/ml (0.2 mM) solution of 3,4-dihydroxybenzoic acid (DHBA). At the end of the incubation time, 0.005 ml of INT (1% in water) was instilled onto the mucus threads which were returned to the Petri dish for 30 min. incubation at 25°C. The reaction was stopped by washing with 50% ethanol/water. The
Fig. 1. In vivo effect of SOD on INT reduction by conjunctival mucus thread. A, Inactivated SOD. B, Active SOD. (×60.)

Fig. 2. Effect of SOD and DHBA on INT reduction by isolated conjunctival mucus. A, Inactivated SOD. B, Active SOD. (×60.)
Fig. 3. Effect of DHBA and NADH on INT reduction by isolated conjunctival mucus. A rather thick piece of mucus thread was cut into four 2 mm square pieces, 0.5 mm thick, and used for the experiment. A, Control. B, DHBA. C, DHBA + NADH+. D, NADH+. Although the color difference is not obvious in these half-tone prints, there is an increase in red-brown oxidation products in C and an increase in violet INT reduction products in D, both produced by NADH addition. (Approx. ×150.)

mucus threads were allowed to dry in place and photomicrographed. To show the effects of nicotinamide adenine dinucleotide, reduced (NADH) on dye reduction, similar preparations were used with the addition of 0.005 ml of a 0.2% solution of NADH (Sigma Chemical Co., St. Louis, Mo.) in water.

Results. SOD destroys superoxide radical by catalyzing the reaction \( \text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O} \). However, NBT reduction by granulocytes can be inhibited by many materials, including autoclaved SOD. For this reason, we used autoclaved SOD as a control to account for any nonspecific effect on INT reduction. (In other studies, we found no apparent difference between autoclaved SOD-treated and non-SOD treated eyes.) Fig. 1, A and B, compare mucus threads from the rabbit treated with INT and native or inactivated SOD in different eyes. A decrease in the amount of INT reduction in the native SOD-treated eyes was seen in all of the animals tested. Like Norn, we found that INT-reducing activity in the mucus thread from sterile eyes was not associated with cells but rather was distributed diffusely throughout the mucus thread and associated with round and oval vacuoles. A large quantitative variability in INT reduction was observed between animals in vivo. Since SOD has powerful anti-inflammatory properties, our quantitatively variable in vivo results could be explicable in terms of interference with the production of the INT-reducing system rather than destruction of superoxide. Thus we also did in vitro studies using a single mucus thread divided into equal sections. Typical results from this work are shown in Fig. 2. As in the in vivo work, there was a decrease in the amount of INT reduction in the presence of native SOD (Fig. 2, A), suggesting a role for the superoxide radical in this reaction.

SOD inhibition of tetrazolium dye reduction has been questioned as an absolutely definitive test for superoxide radical. Furthermore, we found that rather large amounts of SOD were required to produce inhibition. For these reasons, we did another test based upon the fact that the superoxide anion, because of its unpaired electron, is unique in having strong oxidizing as well as reducing properties. The oxidizing reaction has a faster rate than the reducing reaction. Thus good reducing agents such as DHBA or other catechols paradoxically inhibit the reduction of a tetrazolium dye by superoxide by consuming the superoxide radical in a competing oxidation reaction.

This principle has been used to show that the oxygen-dependent reduction of NBT and cytochrome c by photoreduced flavines is a process involving superoxide radical. Similarly, Fig. 3, B shows the inhibitory effect of DHBA on INT reduction by conjunctival mucus. A light reddish brown stain apparent in the DHBA-treated sam-
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Results...