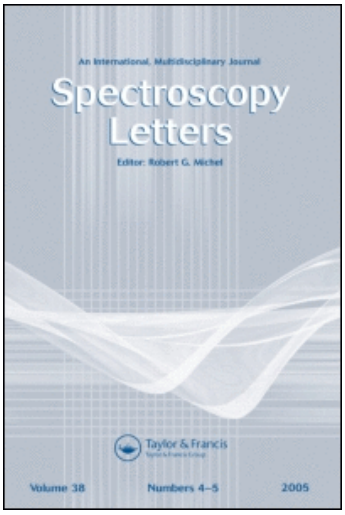

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Quantitative Detection of Superoxide Ions in Whole Blood of the American Alligator (*Alligator mississippiensis*)

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ABSTRACT WST-1 and nitroblue tetrazolium (NBT) were used to quantify production of superoxide ions by leukocytes of the American alligator in whole blood. Because of the difficulty of isolating healthy leukocytes in alligators, these compounds were employed in whole blood instead of leukocyte preparations commonly used in other studies. The detection of superoxide was concentration- and time-dependent and linear with serial dilutions of whole blood. WST-1 reduction was inhibited by superoxide dismutase, indicating that the reduction was due to the presence of superoxide. Light microscopy revealed that superoxide production occurred in leukocytes, with no detectable contributions from other blood components.

KEYWORDS crocodilians, innate immunity, reactive oxygen species, respiratory burst

INTRODUCTION

Production of superoxide is a hallmark of innate immunity. It is the first step in a complex series of enzymatic reactions that characterizes the response to infection and inflammation. When stimulated, leukocytes, via the NADPH oxidase enzyme complex, generate superoxide as a first step in the synthesis of peroxide, hydroxide radical, and hypochlorite. These reactive oxygen species are produced as part of the arsenal used to eradicate potentially pathogenic invading microbes.

WST-1 and Nitrotetrazolium bloe (NBT) have been commonly used as electron receptors for the detection of superoxide. These tetrazolium salts oxidize superoxide to molecular oxygen while being reduced to formazan compounds that exhibit high molar absorptivity coefficients. The tetrazolium compounds are usually used to measure superoxide in leukocyte preparations isolated from whole blood. However, due to the difficulty in isolating healthy leukocytes from crocodilian blood, we have employed these compounds to quantify superoxide produced by leukocytes in fresh, whole, anticoagulated blood from the American alligator.

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MATERIALS AND METHODS

Chemical and Biochemicals

WST-1 was purchased from Roche Diagnostics (Mannheim, Germany). NBT and superoxide dismutase were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Collection of Blood Samples

Blood samples were collected from the spinal vein[1,2] of six alligators using 11/2″ 20-gauge needle and 20-mL syringes. The samples were transferred to Vacutainer™ tubes (Becton-Dickinson, Franklin Lakes, NJ) containing heparin to prevent clotting. The blood samples were pooled for analyses.

Superoxide Assay

A solution of 1 M WST-1 or NBT (0.1 volumes) was added to whole blood for a final concentration of 100 mM. To measure concentration-dependent production of superoxide, whole blood diluted with PBS (pH 7.4) to different cell concentrations was incubated with the WST-1 tetrazolium salt for 4 h at 30°C. The aliquots were centrifuged at $2000 \times g$ for 2 min, and 225 uL of each sample was removed to a single well of a 96-well microtiter plate. The optical density of each sample was determined at 438 nm for samples incubated with WST-1 and 560 nm for samples treated with NBT.

To measure the kinetics of superoxide production, 2 mL 1 M WST-1 or NBT was added to 20 mL whole blood at 30°C. Samples of 500 μL were removed at different time points, the aliquots were centrifuged at $2000 \times g$ for 2 min, and 225 uL of each sample was removed to a single well of a 96-well microtiter plate. The optical density of each sample was determined at 438 nm or 560 nm for samples treated with WST-1 or NBT, respectively.

Statistics and Controls

Samples were analyzed in quadruplicate so that valid statistical analyses could be obtained. The concentrations of superoxide were calculated using 38,500 and 25,400 $M^{-1} cm^{-1}$ as the molar extinction coefficient of the WST-1 (Roche product literature) and NBT[3] formazan products, respectively. All results represent the means ± standard deviations of four independent determinations.

RESULTS

The results displayed in Fig. 1 show the concentration-dependent production of superoxide leukocytes of the American alligator. Incubation of undiluted whole alligator blood with NBT or WST-1 resulted in calculated concentrations of 58.8 ± 3.7 μM superoxide, while 1:2, 1:4, 1:8, and 1:16 dilutions (PBS, pH 7.4) of alligator whole blood (v/v) resulted in 36.6 ± 2.5, 18.9 ± 0.4, 16.6 ± 0.4, and 8.5 ± 0.3 μM superoxide, respectively. The superoxide productions calculated in the same dilutions of alligator blood incubated with NBT were 58.8 ± 3.7, 27 ± 0.6, 13 ± 0.7, 13.5 ± 0.4, and 8.5 ± 0.2 μM,

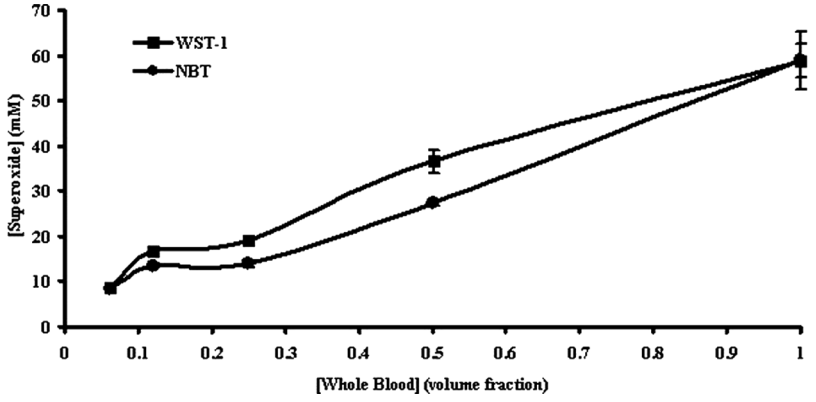

FIGURE 1 **Serial dilutions of fresh whole alligator blood were incubated with 100 mM WST-1 or NBT at 30°C for 4 h. Samples were centrifuged, and the optical densities of the resulting plasma samples were determined at 438 and 550 nm for WST-1 and NBT, respectively, to determine superoxide concentrations. Measurement of superoxide by both WST-1 and NBT result in the concentration-dependent production of superoxide by alligator leukocytes. The results are expressed as the means ± standard deviations for four independent determinations.**

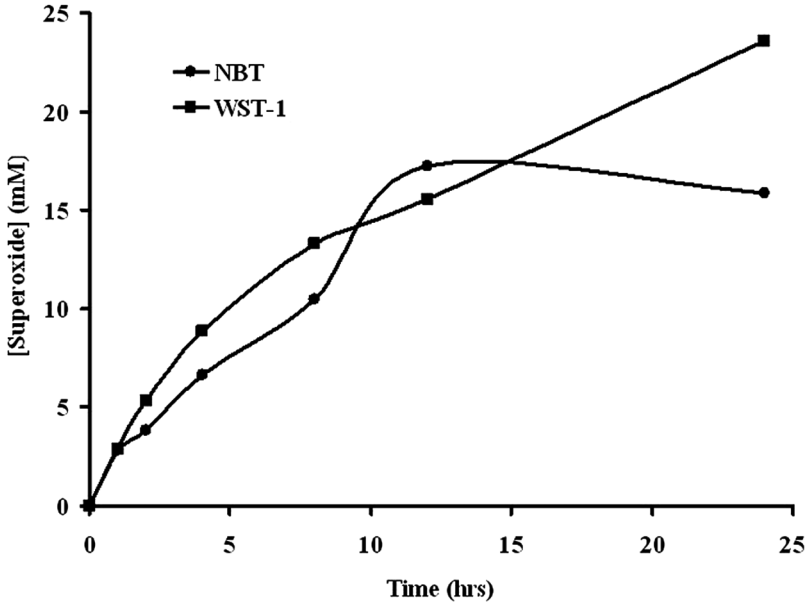

FIGURE 2 **Fresh whole alligator blood was incubated with 100 mM WST-1 or NBT for different lengths of time at 30°C. Aliquots were removed, centrifuged, and the resulting plasma samples were determined at 438 and 550 nm for WST-1 and NBT, respectively, to determine superoxide concentrations. Measurement of superoxide by both WST-1 and NBT result in the time-dependent production of superoxide by alligator leukocytes. The results are expressed as the means ± standard deviations for four independent determinations.**

respectively. In addition, this method proved to be very reproducible, with observed coefficients of variance generally less than 5%.

The results shown Fig. 2 illustrate the time-dependent production of superoxide by alligator leukocytes. Incubation of fresh whole alligator blood with 100 mM NBT or WST-1 resulted in time-dependent production of superoxide. Use of NBT resulted in 2.9, 5.3, 8.8, and 13.3 μM superoxide at 1, 2, 4, and 8 h, respectively. Quantification of superoxide at the same time points using WST-1 resulted in 2.9, 3.8, 6.6, and 10.5 μM superoxide. The values for the two probes are approximately the same at 12 h. However, measurement of superoxide at 24 h using NBT resulted in an increase to 26.5 μM, whereas the use of WST-1 resulted in no

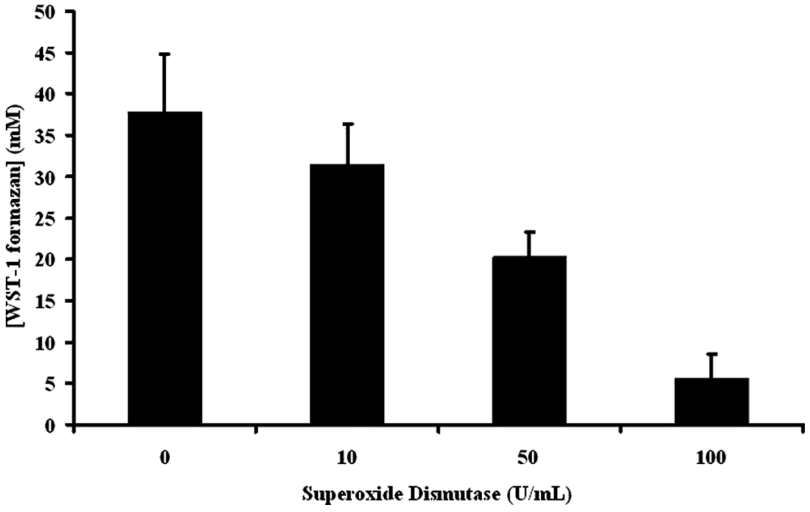

FIGURE 3 **Concentration-dependent inhibition of WST-1 reduction by superoxide dismutase. Fresh whole alligator blood was incubated with WST-1 in the presence of different concentrations of superoxide dismutase. Samples were centrifuged, and the optical densities of the resulting plasma samples were determined at 438 nm to determine superoxide concentrations. The superoxide dismutase reduces the superoxide, produced by the leukocytes, to peroxide, thus inhibiting the reduction of WST-1 in a concentration-dependent manner. The results are expressed as the means ± standard deviations for four independent determinations.**

statistical change ($p > 0.05$) relative to the 12 h time point.

Figure 3 illustrates the effects of superoxide dismutase on the reduction of WST-1 in alligator whole blood. The reduction of the WST-1 tetrazolium salt to the formazan was decreased by $17.0 \pm 4.2\%$, $46.3 \pm 8.1\%$, and $85.2 \pm 7.6\%$ in the presence of 10, 50, and 100 U/mL superoxide dismutase, respectively. The decrease in WST-1 reduction is putatively due to the conversion of superoxide to hydrogen peroxide and molecular oxygen by superoxide dismutase and thus titrating away the superoxide available for WST-1 reduction.

DISCUSSION

Little research has been focused on the immune systems of reptiles. However, understanding reptilian immunology is critical to understanding the evolution of immunity, as reptiles are the progenitors of all avian and mammalian species. Many different phylogenetically diverse species have been shown to produce superoxide in response to infection and inflammation.[4] Mccoll and Daniels[5] demonstrated that leukocytes from the blue-tongue lizard (*Tiliqua scincoides*) produce superoxide, and Terron et al. [6] showed similar activities in ring doves (*Streptopelia risoria*). Other studies have described superoxide production by leukocytes from invertebrate species.[7]. These assays are typically conducted in isolated leukocyte cultures.[8–10]. However, due to the difficulty in isolating healthy leukocyte cultures from alligators, we investigated the possibility of conducting the superoxide assay in whole blood.

The results presented in Fig. 1 clearly show that the dilution of whole blood results in the linear reduction in superoxide detection by both WST-1 and NBT. The two superoxide probes exhibit identical sensitivity in whole, undiluted blood. The slopes of the lines are similar, indicating that both of these superoxide indicators follow similar reduction concentration curves. Unpublished data from phase-contrast microscopy experiments in our laboratory have revealed that the origin of the superoxide measured by WST-1 and NBT is leukocytic in nature. NBT is a yellow, water-soluble tetrazolium salt that is reduced by superoxide to form an insoluble purple formazan dye that is trapped within the cells that produce superoxide.[11] This dye has been used extensively to determine the cellular origin of superoxide in a wide variety of tissues.[12–14] Incubation of whole alligator blood with 100 mM NBT resulted in staining of only heterophils, while other leukocytes, platelets and erythrocytes did not stain, indicating the lack of superoxide production in these cells. These results confirm that the reduction of NBT in the whole blood assay is due to the production of superoxide by leukocytes.[15–17] In addition, incubation of plasma with NBT or WST-1 did not result in the detectable reduction of either salt, indicating that the measured activities were not due to the presence of soluble plasma components.

The data presented in Fig. 2 demonstrate that the time-dependent production of superoxide by alligator leukocytes occurs in a semilinear fashion during a time span of 24 h, as shown by both the WST-1 and NBT molecular probes. However, the detection of superoxide using WST-1 seemed to max at 12 h, as the time point at 24 h resulted in concentrations of superoxide that were not statistically different ($p > 0.05$) than that detected at the 12-h point. Results from trypan dye exclusion[18] showed that the cells were $>97\%$ viable during the course of the experiments (data not shown), and thus the production of superoxide by the leukocytes was not due to nonspecific cell death.

Because it is possible that the reduction of WST-1 and NBT could result due to the presence of other reactive oxygen species, we incubated whole blood with superoxide dismutase. This enzyme catalyzes the conversion of superoxide to peroxide and molecular oxygen. The results in Fig. 3 show that the presence of superoxide reduces the WST-1 reduction in a concentration-dependent manner. Because superoxide is a specific substrate for superoxide dismutase, it is reasonable to assume that the reduction of the WST-1 tetrazolium salt is specifically due to the presence of superoxide and not some other reactive oxygen species.

The data presented in this study clearly show that the production of superoxide can be measured in whole blood without the isolation of leukocytes. In addition, there appears to be no interference or contribution of the superoxide production by blood components other than the leukocytes. This method should be useful for researchers investigating immune mechanisms in species for which the isolation of leukocytes is problematic.

ACKNOWLEDGMENT

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