THE SUPEROXIDE ANION AND SINGLET MOLECULAR OXYGEN: THEIR ROLE IN THE MICROBICIDAL ACTIVITY OF THE POLYMORPHONUCLEAR LEUKOCYTE *

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SUMMARY: Superoxide dismutase was found to partially inhibit both chemiluminescence and nitroblue tetrazolium (NBT) reduction from intact human polymorphonuclear leukocytes. This capacity to reduce NBT was lost when the polymorphonuclear leukocytes were sonicated, but could be regained if exogenous NADPH (or NADH) was added to the system. Superoxide dismutase was found to inhibit this NADPH- and NADH-dependent NBT reduction. A mechanism is proposed that relates superoxide anion generation to the univalent reduction of O_2 by the activated NADPH (and NADH) oxidase. The relationship of superoxide anion production to NBT reduction, singlet molecular oxygen generation, and chemiluminescence is discussed.

I. INTRODUCTION

The PMN leukocyte functions to protect the body through its ability to phagocytize and destroy microorganisms. This microbicidal activity is linked to metabolic alterations involving the mobilization of reducing equivalents and increased non-mitochondrial consumption of O_2 (1). Both alterations appear to result from the phagocytic activation of a granule-bound NADPH oxidase (2). The resultant shift in the NADPH/$NADP^+$ ratio stimulates the hexose monophosphate shunt.

The generation of $\cdot O_2^-$ as a consequence of this metabolic activation has been proposed previously by Allen et al. (3,4),

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Abbreviations are: BSA, bovine serum albumin; SD, superoxide dismutase; MPO, myeloperoxidase; PMN, polymorphonuclear; NBT, nitroblue tetrazolium; CL, chemiluminescence; $\cdot O_2^-$, superoxide anion; $^1O_2$, excited singlet multiplicity molecular oxygen.

and more recently, experimental evidence for its generation has been provided by Babior et al. (5).

Using the enzyme superoxide dismutase, the present investigation was designed to evaluate the role of $\cdot O_2^-$ with respect to NBT reduction and CL. Evidence is presented that $\cdot O_2^-$ is an intermediate in the NADPH (and also the NADH) oxidase reaction.

II. MATERIALS AND METHODS

Human whole blood was obtained by venipuncture. PMN leukocytes were isolated by sedimentation and purified by a modification of the glass bead column method described by Rabinowitz (6,7). The purified cells were suspended in RPMI 1640 tissue culture reagent containing no phenyl red indicator (Associated Biomedic Systems). Total cell counts were made using a hemocytometer, and differential counts were used as an index of preparation purity. Phagocytosis was initiated by the addition of 0.5 ml of autologous serum containing 0.5 mg dry weight of Propionibacterium shermanii.

NBT reduction was monitored using a modification of the method described by Baehner and Nathan (8). The blue formazan precipitate was extracted with pyridine and the absorbance of the extract was determined using a Beckman DU spectrophotometer at 515 nm against a pyridine blank.

Chemiluminescence was monitored with a Packard Scintillation Spectrometer Model 3320 operated in the out-of-coincidence summation mode. The modified Model 3320 served as an ultrasensitive photomultiplier, and the monitored CL was considered to reflect energy transductions resulting in the population of electronic excitation states that relax with photon emission. The instrument was operated at 22°C using

the window settings 0 - ∞ and at 100% gain.

Superoxide dismutase was purchased from Miles Laboratories (2400 units/mg) and Sigma Chemical Company (2900 units/mg). NBT, NADH, NADPH, and BSA were obtained from Sigma Chemical Company. Other chemicals used were of reagent grade and solvents were of spectral grade.

III. RESULTS

Temporal traces of CL obtained from phagocytically challenged PMN leukocytes in the presence of either SD or BSA are shown in Figure 1. Superoxide dismutase was found to inhibit both CL and NBT reduction, and increasing the concentration of SD in the incubating media increased this inhibition. However, very high concentrations of SD were not found to inhibit proportionately CL or NBT reductions, and this might

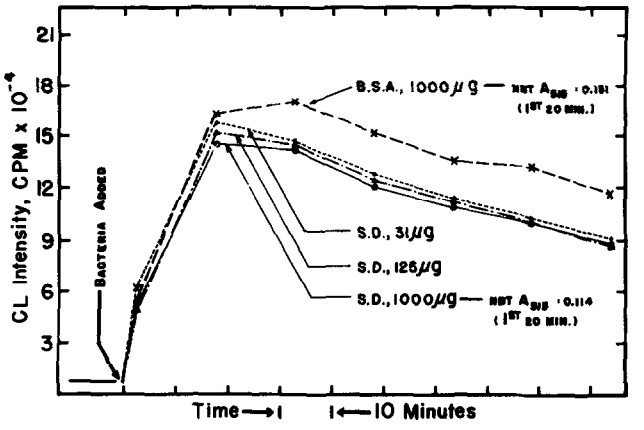

Figure 1. Comparison of temporal traces of CL in the presence of BSA versus varying concentrations of SD (Miles Lab.) as emitted by phagocytically challenged PMN's. System: 6 x 10^6 PMN leukocytes (95% purity) suspended in 2.5 ml RPMI 1640 buffer were incubated with the BSA or SD for two minutes prior to challenge with P. shermanii (0.5 mg dry weight opsonized in 0.5 ml autologous serum). To identical systems NBT was added 6 minutes following initiation of phagocytosis, and the absorbance at 515 nm of the reduced NBT (formazan) was determined 20 minutes after the addition of NBT.

reflect the difficulty of SD getting to the site of $\cdot O_2^-$ generation. Very recently, Webb et al. have reported the SD-dependent inhibition of CL by as much as 70% with 100 μg/ml of the enzyme (22). In our hands inhibition no greater than 30% was observed with SD obtained from either Miles Laboratories or Sigma Chemical Co. However, these investigators used opsonized zymosan particles to initiate phagocytosis. Opsonized bacteria (P. shermanii) were used in the experiments reported in this publication.

Figure 2 shows the results of SD-mediated inhibition of NBT reduction by challenged PMN leukocytes over varying periods of NBT incubation. This inhibition in reduction is consistent with the findings of Beauchamp and Fridovich who reported that NBT reduction by a xanthine oxidase system can be inhibited by SD in an aerobic assay system by as much as 90% (9).

If NBT reduction is mediated through $\cdot O_2^-$ generation in

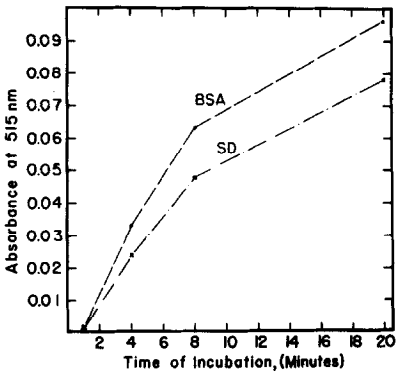

Figure 2. Comparison of NBT reduction, as measured by absorbance at 515 nm, of BSA systems versus SD systems at varying time intervals after bacterial challenge. System: 1.0 x 10^7 PMN leukocytes (95% purity) in 2.5 ml RPMI 1640 buffer were incubated with 250 μg BSA or SD (Miles Lab.) for 2 minutes. Phagocytosis was initiated by P. shermanii (0.5 mg dry weight opsonized in 0.5 ml autologous serum) and allowed to continue for varying intervals of time before killing.

the PMN leukocyte, the possibility exists to investigate the inhibitory effects of SD on the flavoprotein oxidase systems responsible for reduction. Figure 3 presents the results of an experiment designed to compare the capacity of resting and phagocytizing PMN leukocytes to reduce NBT. Note that SD consistently inhibited NBT reduction in all systems investigated. Sonication of either resting or phagocytizing PMN leukocytes resulted in inhibition of activity to below resting levels. However, if NADPH or NADH was added to the previously challenged sonicated PMN leukocytes, activity was restored. NADPH

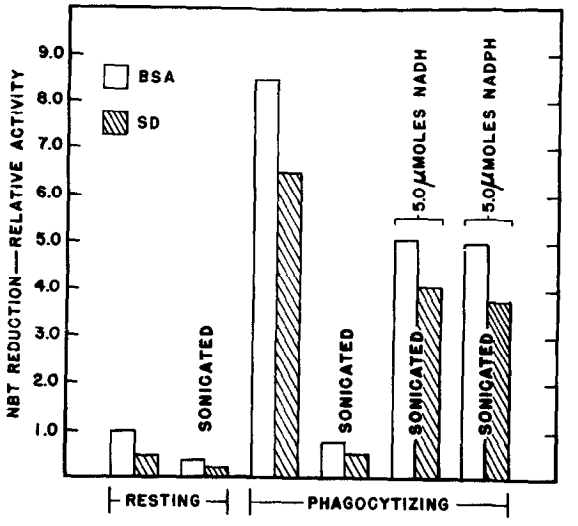

Figure 3. Relative activities of BSA systems versus identical SD systems; comparing the effects of phagocytosis, sonication, and addition of NADH or NADPH. The relative activities are a result of the ratio of the absorbance (at 515 nm) of the formazan extract of each particular BSA or SD system to the absorbance of the resting unsonicated BSA system (A_{515}: 0.013) (the activity of this resting, unsonicated BSA system therefore being equal to 1.0). System: 1.5 x 10^7 PMN leukocytes (97% purity) in 2.5 ml RPMI 1640 buffer were incubated with 500 μg BSA or SD (Miles Lab.) and P. shermanii (0.5 mg dry weight opsonized in 0.5 ml autologous serum) for 3 minutes. Appropriate samples were then sonicated in ice for 2 minutes, whereupon 5.0 μ moles NADH or NADPH (where applicable) were added. NBT (0.5 ml of 0.1% solution in saline) was then added to all systems and allowed to incubate at 37° C for 20 minutes.

and NADH associated activity showed a similar inhibition in the presence of SD. No increase in activity was observed when NADPH was added to previously challenged unsonicated PMN leukocytes.

The addition of NBT to challenged unsonicated PMN was also found to decrease the CL response by approximately 70%. This finding suggested the loss of reducing potential to NBT with a resulting decrease in $\cdot O_2^-$, HOOH, and $^1O_2$ production, and therefore a decrease in CL. An alternate explanation is that the colored formazan precipitate formed may physically absorb the emitted CL.

IV. DISCUSSION

The microbicidal metabolism of the PMN leukocyte has recently become the subject of certain interesting observations concerning the univalent reduction and electronic excitation of O_2, and the participation of these species in microbicidal activity (4,5). In a more general sense, such observations open new avenues of approach to the study of bioenergetics, namely the possibility for investigation of the biological quantum manipulation of a molecule. Figure 4 is a schematic attempt to integrate these observations into a self-consistent unified metabolic sequence.

The PMN leukocyte responds metabolically to phagocytosis by increased generation of NADPH via the hexose monophosphate shunt, and increased non-mitochondrial O_2 consumption. Both events have been shown by Rossi *et al.* (2) to reflect the activation of the flavoprotein NADPH oxidase. Figure 3 shows a corresponding activation of NADH oxidase, a finding in accord with the reports of numerous investigators (10,11,12). However, the *in vivo* significance of NADH oxidase activity has been questioned (2).

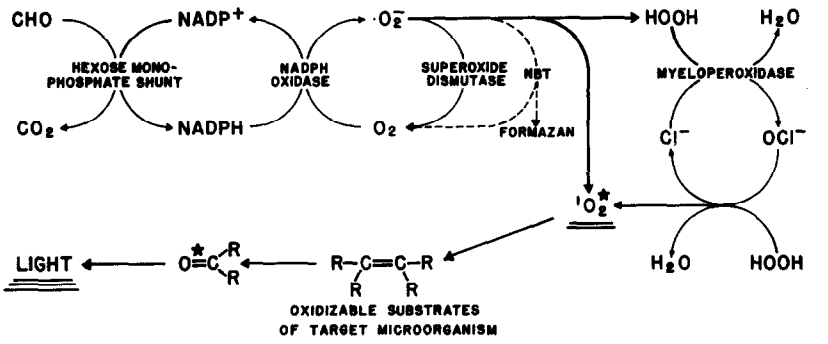

Figure 4. Schematic of a proposed mechanism to describe the microbicidal metabolism of the PMN leukocyte and the action of both SD and NBT on this system.

Reasoning that many flavoprotein oxidases of this type univalently reduce O_2 to $\cdot O_2^-$, Allen et al. proposed that these oxidases might function in the generation of $^1O_2$, either directly or indirectly (3). Direct generation of $^1O_2$ can result from the nonenzymatic disproportionation of $\cdot O_2^-$ in solutions of high hydrogen ion concentration as found in the phagolysosome (13) through the mechanism proposed by Stauff et al. (14).

$$\cdot O_2^- + HO_2 \xrightarrow{H^+} {}^1O_2 + HOOH \qquad (1)$$

The direct generation of $^1O_2$ involves the HOOH product of Eq. 1 that is now available to the MPO - Halide - HOOH microbicidal system described by Klebanoff (15). This isolated system has been shown to chemiluminesce and $^1O_2$ has been proposed as the microbicidal agent (7,16). Furthermore, Klebanoff has been able to inhibit the microbicidal activity of the system using the $^1O_2$ trap, DABCO (1,4-diazobicyclo [2,2,2] octane) (17).

$$2\ HOOH \xrightarrow[Cl^-]{MPO} {}^1O_2 + 2\ H_2O \qquad (2)$$

With regard to inhibition of CL, Arneson has proposed that SD prevents the formation of $^1O_2$, as described in Eq. 1, by re-

leasing triplet multiplicity ground state 0_2 and HOOH as the products of dismutation (18).

$$2 \cdot O_2^- \xrightarrow{SD} HOOH + {}^3O_2 \text{ (Ground state)} \qquad (3)$$

Finazzi Agro et al. (19) have also proposed that SD can relax $^1O_2$ directly to its ground state, and can thereby inhibit $^1O_2$-mediated oxidation. Recently, evidence has been presented demonstrating that SD does not function to relax $^1O_2$ directly (20).

According to the proposed mechanism, SD would not be expected to totally block the generation of $^1O_2$, but would be responsible for the inhibition of CL related to $\cdot O_2^-$ disproportionation to $^1O_2$ and HOOH. The residual HOOH is still available to react with the MPO - Halide - HOOH system in the generation of $^1O_2$. This may be related to the inability to inhibit CL past a certain point regardless of the quantity of SD added.

The generated $^1O_2$ may react with a wide spectrum of substrates present in the target microorganisms and thus serve as an oxidative microbicidal agent (21). The $n\pi^*$ electronically excited carbonyl products of $^1O_2$-mediated oxidations are capable of relaxation to ground state by emitting photons (23). It is also possible that these excited carbonyls react with substrates on the target microorganisms, and participate in microbicidal activity. Although relaxation of excited carbonyls is the more probable source of CL, the possibility exists that CL results from the pooled relaxation of $^1O_2$ to ground state; however, such a relaxation would reflect poor energetic economy.

In summary, the microbicidal bioenergetics of the PMN leukocytes involves energy transductions in which the covalent bond energy of glucose is mobilized in the form of reducing equivalents. These equivalents are then available to drive

electronic manipulations that result in the generation of electronically excited molecules, such as $^1O_2$ and excited carbonyl groups, that can relax by photon emission.

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