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THE USE OF WATER-SOLUBLE RADICAL SCAVENGERS TO DETECT HYDROXYL RADICAL FORMATION BY POLYMORPHONUCLEAR LEUKOCYTES

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The polymorphonuclear leukocyte secretes both O_2^- and H_2O_2 when stimulated by various soluble or particulate stimuli. Since a reaction involving iron, O_2^-, and H_2O_2 could generate the hydroxyl radical ($HO\cdot$) there has been speculation that the $HO\cdot$ may participate in the bactericidal activity of the neutrophil. A variety of water-soluble $HO\cdot$ scavengers have been used to test for the participation of $HO\cdot$ and the results imply that $HO\cdot$ might participate. However, other workers have not been able to detect the formation of significant amounts of $HO\cdot$ by the activated neutrophil. We have examined the effect of several commonly used $HO\cdot$ radical scavengers on the ability of the neutrophil to secrete O_2^- and H_2O_2. Several of these compounds actively inhibit secretion without affecting the viability of the neutrophil. After considering the various complications inherent in using water soluble radical scavengers, we suggest that they only be used with well defined experimental systems.

KEY WORDS: Radical scavengers, hydroxyl radical, polymorphonuclear leukocytes, superoxide, hydrogen peroxide.

ABBREVIATIONS: Dimethylsulfoxide, DMSO; dimethylthiourea, DMTU; lactic acid dehydrogenase, LDH; phorbol myristate acetate, PMA; polymorphonuclear leukocyte, PMN; superoxide dismutase, SOD.

INTRODUCTION

The polymorphonuclear leukocyte (PMN) is one of the main defenses that the body has against invading bacteria and yeast. After the microbes are phagocytosed a variety of enzymes and other chemicals (including reduced oxygen species) are secreted into the phagolysosome. The PMN has a complex enzyme system that utilizes electrons derived from the hexose monophosphate shunt to reduce oxygen to superoxide (O_2^-).[1,2] Both O_2^- and H_2O_2 are detected in the medium[1,2] after the PMN is activated. A substantial fraction of the H_2O_2 is consumed by myeloperoxidase, which constitutes approximately 5% of the PMN dry weight,[3] to produce HOCl.

In the presence of transition metals like iron, O_2^- and H_2O_2 can participate in a Fenton type reaction that yields hydroxyl radical ($HO\cdot$). Because $HO\cdot$ is a much more powerful oxidant than either O_2^- or H_2O_2 and because it can initiate free radical autoxidation, it has been assumed that $HO\cdot$ could play a major role in oxidative reactions of the PMN. Several different water-soluble compounds that scavenge $HO\cdot$ have been added to solutions of activated PMNs to test for the presence of $HO\cdot$. This

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has been done despite reports that many of these compounds also react with HOCl. An additional complication can arise if these compounds were to interfere with the mechanism of oxidant generation by the PMN. We present evidence that several of the reagents commonly used to test for the participation of HO· lower the secretion of O_2^- and H_2O_2 without affecting PMN viability as measured by the release of lactate dehydrogenase (LDH) into the medium.

MATERIALS AND METHODS

Human PMN were isolated as previously reported.[5] The release of O_2^- was measured from the superoxide dismutase (SOD) inhibited reduction of cytochrome c[I] (Sigma Chemical Co.) measured at 550 nm after pelleting the PMNs or by monitoring the absorbance increase of the α-band (550 nm) with respect to the isobestic point at 556 nm using a mean difference ε of $1.35 \times 10^4 M^{-1}cm^{-1}$. Absorption measurements were made on a Hewlett Packard Model 8452A spectrophotometer. The release of H_2O_2 was measured with a H_2O_2 electrode and Model 25 oxidase meter from Yellow Springs Instrument Co. Experiments were performed at 37°C with 5×10^6 PMN/mL, 5.5 mM glucose, in Hanks' Balanced Salt Solution (HBSS, GIBCO Laboratories) containing 4.2 mM sodium bicarbonate (pH 7.4), and various scavengers to give a final volume of 3 mL. When H_2O_2 was measured the solutions contained 10 mM sodium azide to inhibit myeloperoxidase. All reactions were initiated by the addition of phorbol myristate acetate (PMA, 33 ng/mL final concentration). The rate of cytochrome c reduction was made between 3 and 8 min where the rate of reduction was linear. In some cases the H_2O_2 measurements were obtained 15 min after stimulation by pouring the tube contents into a thermostated electrode cell and recording the signal for the next 2 min. In other cases the quantities were obtained by running the experiment for 30 min and comparing the rate of H_2O_2 release at 15 min. The relative amount of LDH was determined by measuring the consumption of NADH using literature procedures.[4] The value obtained when the cells were digested with Triton X-100 (Sigma Chemical Co.) was used to represent 100% of the cellular LDH.

RESULTS

Six compounds were studied for their effect on the reduction of cytochrome c by PMN stimulated with PMA. SOD was used to differentiate the reduction by O_2^- from other reduction processes. Of these compounds, mannitol, dimethyl sulfoxide (DMSO), benzoate, and dimethyl thiourea[5,6] are routinely[6,7] used to scavenge HO· in biological systems. Thiourea is a good radical scavenger,[5] but it was expected to be somewhat toxic. Allopurinol was included as an example of how a xanthine derivative might affect O_2^- secretion. The highest concentrations used in this study were the highest concentrations reported in the literature for the use of these compounds as HO· scavengers. Table I shows the effect of each scavenger on the reduction of cytochrome c. When 27 μM reduced cytochrome c (ferrocytochrome c) was treated with 18 μM H_2O_2, it was not oxidized to ferricytochrome c.

An H_2O_2 electrode was used to measure the effect of four of the scavengers on the secretion of H_2O_2 by the PMN. Dimethylthiourea caused a sharp change in the base

TABLE I

The effect of several water-soluble hydroxyl radical scavengers on the secretion of superoxide and hydrogen peroxide by the human polymorphonuclear leukocyte.

Name	Concentration (mM)	Cytochrome c reduction[a] % of control	H_2O_2 formation[a] % of control
Allopurinol	0.69[b]	74 ± 8	70 ± 9
Benzoate	36	97 ± 8	71 ± 2
	72[b]	63 ± 6	59 ± 22
	150	60 ± 23	-----
DMSO	117[b]	94 ± 4	89 ± 5
DMTU	50	99 ± 16	-----
	75	79 ± 11	-----
	100[b]	33 ± 20	-----
Mannitol	50	81 ± 2	82 ± 15
	100[b]	67 ± 1	68 ± 13
Thiourea	1	100 ± 5	-----
	10[b]	70 ± 16	-----

[a] These numbers are derived by dividing the value obtained for experiments performed in the presence of scavenger by the value obtained in the absence of scavenger. Each experiment has been repeated from 2 to 7 times with at least 2 determinations for each experiment. The procedures are described in the Materials and Methods.

[b] Scavenger concentrations examined for LDH release.

line current for the H_2O_2 electrode and was not further studied by this method. Table I shows the effect of allopurinol, benzoate, DMSO and mannitol on the production of H_2O_2 by PMA stimulated PMNs.

When LDH release was measured for various concentrations of allopurinol, benzoate, DMTU, mannitol and thiourea it was found the percent of LDH release was within experimental error of the value found in the absence of these chemicals (data not shown). These studies were carried out in duplicate with and without added PMA.

To determine how H_2O_2 and hypochlorite affect the function of the scavengers several of the compounds were incubated with these oxidants. Solutions 94 μM in H_2O_2 were monitored with an H_2O_2 sensitive electrode and various concentrations of the scavengers were added to the cell. Both mannitol and DMSO were found to be ineffective in causing the decomposition of H_2O_2 at concentrations that were used with the PMN. The other scavengers were not examined. The reaction of hypochlorite with the scavengers was studied by monitoring the decay of hypochlorite (292 nm) injected into a cuvette (1 to 5 mM final concentration) containing each of the scavengers. Thiourea, DMTU and DMSO had finished reacting within the time of mixing ($k > 13\,M^{-1}s^{-1}$). Benzoate did not react while allopurinol and mannitol reacted with rate constants of $0.22\,M^{-1}s^{-1}$ and $0.04\,M^{-1}s^{-1}$, respectively.

DISCUSSION

The generation of O_2^- as measured as the SOD inhibitable reduction of cytochrome c was strongly affected by several of the scavengers: benzoate at concentrations greater than 50 mM; DMTU at concentrations greater than 75 mM; and thiourea at concentrations greater than 1 mM. However, at the highest concentrations tested

these reagents did not cause an increase in extracellular LDH when compared to control PMNs that were treated in the same manner in the absence of scavenger. Therefore, it is obvious that these reagents are disabling the synthesis of O_2^- without damaging membrane integrity.

Although the specific mechanisms that cause inhibition of both O_2^- and H_2O_2 secretion have not been elucidated in this study, these results demonstrate that with the exception of DMSO the compounds used as radical scavengers modulate the secretion of H_2O_2 and O_2^-. Since the yields of both are affected in roughly the same proportion, it is likely that the effect of the scavengers is to retard the secretion of O_2^- by inhibiting the activity of the PMN, NADPH-oxidase.

There have been a number of questions raised concerning the meaning of obtained data obtained utilizing water-soluble HO· scavengers.[7,8] Recently, four groups[9-13] have reported that the formation of HO· could not be detected in PMN preparations and Winterborn[4] has noted that PMN-myeloperoxidase could consume a large fraction of the H_2O_2 generated by the PMN. Therefore, it is possible that only a small amount of H_2O_2 is available to participate in Fenton chemistry and HOCl (or a related derivative) is the major oxidant. Several years ago Albrich *et al.*[14] pointed out that HOCl could oxidize a wide variety of biologically important molecules. Not unexpectedly, our preliminary results with NaOCl oxidation of the HO· scavengers show that all of the scavengers except benzoate are oxidized by NaOCl at pH 7.6. In light of our results and the results of others, the use of most soluble HO· scavengers should be suspect since many of these scavengers can inhibit both O_2^- and H_2O_2 formation and/or react with HOCl thereby giving the appearance of having scavenged HO·.

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