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Research Article

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Evidence for Hydroxyl Radical Generation by Human Monocytes

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ABSTRACT A number of highly reactive oxygen species have been implicated in the oxygen-dependent mechanisms involved in bactericidal activity of phagocytic leukocytes. Hydrogen peroxide and superoxide, two agents known to occur during phagocytosis, are thought to interact to generate hydroxyl radical, singlet oxygen, and other potentially reactive molecules. Using an assay system of ethylene generation from methional, cell preparations of human monocytes were demonstrated to generate hydroxyl radical or a similar agent during phagocytosis of zymosan particles. The generation of ethylene was impaired by agents which reduce superoxide or hydrogen peroxide concentrations as well as by agents reported to be hydroxyl radical scavengers. The ethylene generation did not appear to be dependent on myeloperoxidase in that azide enhanced ethylene generation. Monocytes from a patient with chronic granulomatous disease failed to generate ethylene during phagocytosis. This assay technique may be useful in exploring the metabolic events integral to the bactericidal and inflammatory activity of phagocytic leukocytes.

INTRODUCTION

Phagocytic cells exposed to an atmosphere of nitrogen demonstrate impaired antimicrobial activity despite normal phagocytosis (1). Thus, it appears that oxygen plays an integral role in the subsequent events leading to death of the ingested organism. Similarly, patients with chronic granulomatous disease (CGD)[1] have leukocytes which have defective bactericidal function and no burst in oxygen consumption associated with phagocytosis (2, 3). The oxygen-related agents implicated in the bactericidal event include superoxide anion ($O_2 \cdot^-$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH \cdot$), singlet oxygen (O_2^1), and potentially other oxygen species (4). It is hypothesized that these agents are produced at the cell surface after membrane pertubation. Several recent studies have demonstrated that human monocytes produce $O_2 \cdot^-$ and, thus, H_2O_2 during phagocytosis (5, 7). Recent indirect evidence has demonstrated the possible involvement of $OH \cdot$ or similar radicals in the microbicidal event and as a mediator of inflammation (8–10). The hydroxyl radical is one of the strongest oxidizing agents known and its existence as a highly reactive transient generated during phagocytosis would be a powerful addition to the leukocyte armamentarium.

In 1970, Beauchamp and Fridovich demonstrated the generation of $OH \cdot$ by a cell-free xanthine-xanthine oxidase system (11). When the enzymatic oxidation of xanthine took place in the presence of methional (β-methyl-thipropionaldehyde), ethylene gas (C_2H_4) was generated. They proposed that $O_2 \cdot^-$ and H_2O_2, two agents known to be generated by the enzymatic system, interacted via the Haber-Weiss reaction to produce the $OH \cdot$ (12). The $OH \cdot$, thus generated, could oxidize methional with C_2H_4 as one of the products whose generation can be quantitated as outlined below.

$$H_2O_2 + O_2^{\cdot -} \rightarrow OH^{\cdot} + OH^- + O_2.$$

$$OH^{\cdot} + \underset{\text{methional}}{HCOCH_2CH_2SCH_3} \rightarrow \underset{\text{ethylene}}{C_2H_4} + \tfrac{1}{2}(CH_3S)_2 + HCOOH.$$

The validity of this scheme would depend upon a requirement for $O_2 \cdot^-$ and H_2O_2 as well as inhibition of ethylene production by agents known to scavenge $OH \cdot$. Superoxide dismutase (SOD) and catalase are enzymes capable of reducing $O_2 \cdot^-$ and H_2O_2, respectively. Beauchamp and Fridovich demonstrated that both of these enzymes inhibited C_2H_4 production as did the two $OH \cdot$ scavengers, benzoate and ethanol

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[1] *Abbreviations used in this paper*: CGD, chronic granulomatous disease; SOD, superoxide dismutase.

(11). Therefore, this system appeared to provide a means of recognizing OH· generation in a cell-free system. We have examined its applicability to more complex intact phagocytic cells, i.e. human blood monocytes.

METHODS

Human monocytes were studied in mononuclear cell preparations obtained by Ficoll-Hypaque (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) technique from the peripheral blood of normal volunteers (13). Cells were typically greater than 95% viable as judged by trypan blue exclusion and contained 25–30% monocytes, 70–75% lymphocytes, and less than 2% polymorphonuclear leukocytes. C_2H_4 generation was determined by incubating the cell preparations in Selegman's and Hanks' balanced salt solution (pH 7.4) with 1 mM methional (Sigma Chemical Co., St. Louis, Mo.) in a final volume of 3.0 ml at 37°C. The reactions took place in sealed, siliconized glass tubes for 20 min with or without opsonized zymosan as the ingested particle. Opsonized zymosan was prepared by incubating 1 vol of zymosan in normal saline with 3 vol of human serum (stored at −80°C) for 30 min at 37°C. After incubation, the particles were washed and resuspended to the original concentration of 50 mg/ml. Other additions included bovine SOD (3000 μ/mg, Sigma Chemical Co.), catalase (C-40, Sigma Chemical Co.), sodium azide (Fisher Scientific Co., Fair Lawn, N. J.), and *N*-ethylmaleimide (Eastman Kodak Co., Rochester, N. Y.). Catalase demonstrated no SOD activity as determined by the method of McCord and Fridovich (14) nor did it inhibit nitroblue tetrazolium reduction by phagocytizing monocytes. Scavengers of OH· were added in concentrations which produced maximal inhibition without adverse effect on cell viability or phagocytic rate (15). Reactions were terminated by the addition of ice cold minimal essential medium at a final concentration of 1 mM injected through the rubber stopper. 1-ml aliquots of the vapor phase were analyzed on a packard 602 flame ionization gas chromatograph (Packard Instrument Co., Inc., Downers Grove, Ill.). The chromatograph was equipped with a 120 cm × 3 mm stainless steel column packed with alumina. Gas flow rates were 300 ml/min air, 25 ml/min hydrogen, and 25 ml/min nitrogen with the injector, detector, and column of 110°, 150°, and 85°C, respectively. Standardization and quantitation of ethylene with this system have been previously described (15). Neither monocyte-depleted preparations of mononuclear cells (obtained by incubation of blood with iron particles before Ficoll-Hypaque separations [16]) or platelets in concentrations comparable to those contaminating our preparations (isolated by differential centrifugation) were capable of generating detectable amounts of C_2H_4 in the absence or presence of opsonized zymosan. 1 mM methional did not alter monocyte viability or phagocytic rate.

RESULTS

Fig. 1A depicts a representative study of C_2H_4 generation as a function of time. The majority of C_2H_4 was produced during the first 20 min after phagocytosis. Fig. 1B illustrates the linear relationship of cell number to C_2H_4 generation.

As summarized in Table I, a low amount of C_2H_4 was detected in preparations of resting monocytes whereas a dramatic increment in C_2H_4 was noted during phagocytosis. No C_2H_4 was detected if methional was deleted from the incubation system. In a study of monocytes from a patient with CGD, the low resting

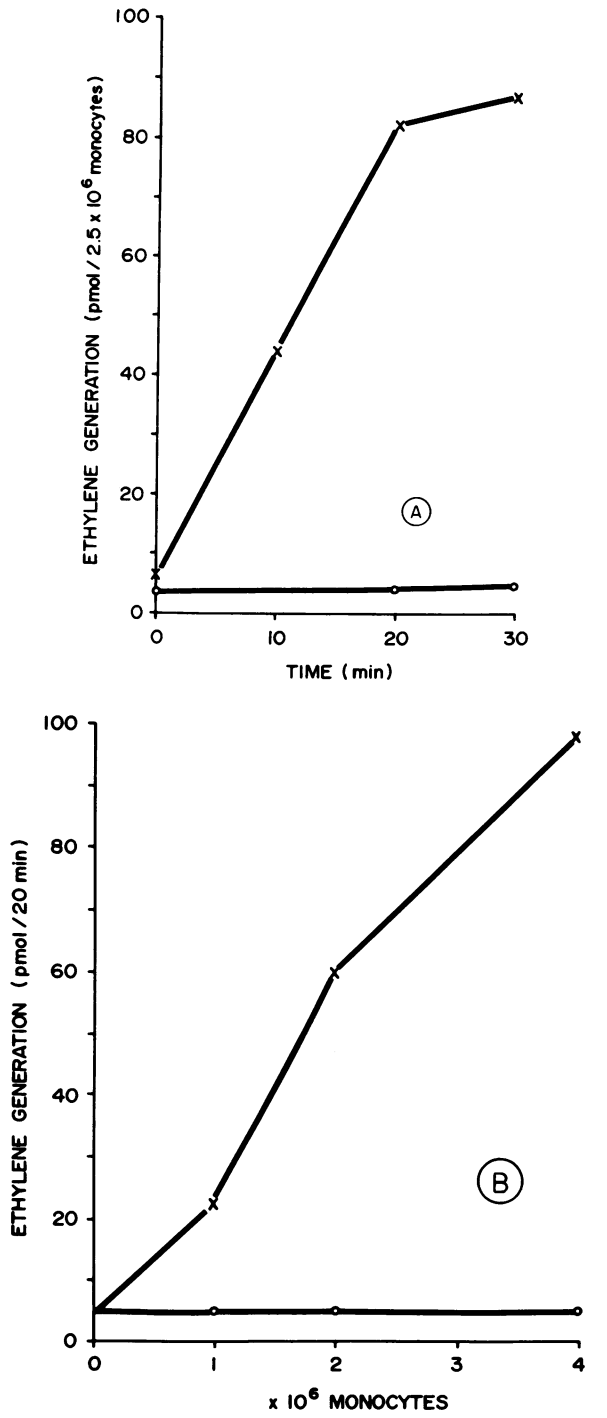

FIGURE 1 The generation of C_2H_4 by 2.5 × 10^6 monocytes as a function of time (A), and the effect of varying monocyte number on C_2H_4 generation (B). Incubations were carried out with 2.5 mg of opsonized (– × – × – × –) or nonopsonized (– O – O – O –) zymosan.

TABLE I
Ethylene Generation by Monocytes

	pmol
Resting monocytes (n = 6)	3.6±0.3*
Phagocytizing monocytes (n=6)	82.3±33.0
Resting CGD monocytes (n = 1)	1.4
Phagocytizing CGD monocytes (n = 1)	1.4

* Mean±1 SD pmol/2.5 × 10^6 monocytes in 20 min.

value of C_2H_4 generation was not enhanced by the addition of opsonized zymosan particles (Table I).

If the Haber-Weiss reaction (12) describes the scheme responsible for OH· production in phagocytic monocytes, SOD and catalase should inhibit C_2H_4 production. SOD inhibition was 91% where as catalase inhibition was 55% (Table II). Heat-inactivated enzymes did not effect C_2H_4 generation. Albumin, at a concentration of 250 μg/ml, inhibited C_2H_4 generation 12±4%. This small affect may be due to its mild ability to scavenge the OH·. Finally, if the OH· is the mediator of C_2H_4 generation from methional, one would expect inhibition by compounds known to scavenge this radical (17). Indeed, 20 mM benzoate inhibited 45±14%, 40 mM ethanol inhibited 46±7%, and 1 mM tryptophan inhibited 92±6%. These concentrations of inhibitory enzymes or agents caused no decrease in viability or phagocytosis as assayed by the Oil Red O system of Stossel (18).

Myeloperoxidase, an enzyme found within monocytes and neutrophils, has been demonstrated to have a potent microbicidal activity in the presence of H_2O_2 and a halide (19). To rule out the possibility that this enzymatic reaction was the source of ethylene generation, experiments were performed in the presence of azide, a potent inhibitor of myeloperoxidase-mediated microbicidal capacity (20). 0.1 mM azide increased C_2H_4 production 73±30% (n = 4). This effect may be due to increased levels of intracellular H_2O_2 secondary to the inhibition of myeloperoxidase or catalase.

TABLE II
Specificity Studies

	Dose	Inhibition*
		%
Catalase (n = 4)	250 μg/ml	55±13
SOD (n = 4)	250 μg/ml	91±11
Benzoate (n = 4)	10 mM	30±8
Benzoate (n = 3)	20 mM	45±14
Ethanol (n = 3)	20 mM	31±6
Ethanol (n = 4)	40 mM	46±7
Tryptophan (n = 4)	1 mM	92±6

* Mean inhibition±1 SD.

DISCUSSION

In this study, monocytes ingesting particles were found to generate C_2H_4 from methional, a reaction thought to reflect OH· generation in a cell-free system (11). This generation occurred primarily in the first 20 min after initiation of phagocytosis, which is a similar time course for phagocytosis-induced generation of O_2^-, H_2O_2, and chemiluminescence by human monocytes (5, 6). Further, C_2H_4 generation was dependent on both O_2^- and H_2O_2, suggesting that the Haber-Weiss reaction (12) may describe the scheme responsible for OH· production in these cell preparations.

Although the detection of C_2H_4 provides evidence for OH· generation, some other alternative oxygen radical might be responsible for ethylene generation. However, the amounts of OH· or similar agent detected by this assay represent a minimal value. This highly unstable radical (OH·) has the capacity to react with a myriad of intracellular compounds as well as the ingested particle so that methional must compete with a variety of concurrent reactions capable of scavenging OH· (21). The use of OH· scavengers requires limited interpretation especially with regard to intact cell systems where oxygen radicals are apparently generated into both the extracellular milieu and intracellular phagosome. Thus, the degree of inhibition seen with chemical scavengers of OH· will reflect in part their rate constant of interaction as well as their ability to gain access to the sites oxygen radical generation. Thus, the ability of tryptophan to almost completely inhibit C_2H_4 generation may reflect either its rate constant of interaction, which is approximately twice that of benzoate and 10 times that of ethanol (17, 21), or some other characteristic which allows greater access or availability at the site of OH· generation (cell membrane or intracellular phagosome). Thus, the observation of Johnston et al. that OH· scavengers produce only a modest impairment of leukocyte bactericidal activity (8) should not be used to minimize the potentially important role of this radical. Indeed, the ability of both catalase and SOD to inhibit bactericidal activity (8) implies a potential role of radicals generated from the interaction of O_2^- and H_2O_2 in the bactericidal event. McCord and Salin have proposed an inflammatory role for OH· derived from leukocytes using similar experimental observations (9, 10). Using this ethylene assay, we have recently provided evidence for OH· generation by human blood granulocytes during phagocytosis (unpublished observations) indicating that this radical may be a common product of phagocytic cells.

This study thus suggests that ethylene generation from methional represents an assay system which provides a highly sensitive means of detecting OH· production in phagocytic cell systems. It also offers

direct evidence that human blood monocytes generate O_2^- and H_2O_2 which interacts to produce OH· or similar oxygen metabolite during ingestion of particles. The role of OH· generation in mediating bactericidal and inflammatory effects of phagocytic leukocytes requires additional exploration.

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