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Production of Hydroxyl Radical by Human Alveolar Macrophages

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Stimulated human alveolar macrophages were demonstrated to oxidize B-methyl proprionaldehyde (methional) or 2-keto-4-thiomethylbutyric acid to ethylene (C_2H_4). Agents which are believed to scavenge the hydroxyl radical (·OH), sodium benzoate, and mannitol, as well as scavengers of superoxide anion (O_2^-) or hydrogen peroxide, decreased C_2H_4 production, implicating ·OH as the oxidizing radical. Differences in C_2H_4 production, as well as oxygen uptake and O_2^- release between human alveolar macrophages and polymorphonuclear leukocytes, were also documented.

After stimulation by bacteria, phagocytes produce reactive species of partially reduced oxygen (O_2), which are believed to be important for killing ingested microorganisms (17). This assumption is supported by impaired bactericidal capabilities of polymorphonuclear leukocytes (PMN) from patients with chronic granulomatous disease, whose phagocytes fail to generate reactive O_2 species, and by the decreased bactericidal activities of normal cells treated with agents which scavenge these O_2 intermediates (12, 15, 19). The exact nature and role of the O_2 products produced by various phagocytes is unknown, but it is believed that superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), or singlet oxygen ($^1O_2$) may be involved in the bactericidal activities (1, 2, 14-16, 18). In addition, leakage or secretion of certain of these agents into the extracellular fluid may be toxic to several types of mammalian cells (19, 25). It has been shown that human PMN and monocytes (MN) make O_2^- and H_2O_2 (1-3, 12). Mechanisms for formation of ·OH and other O_2 intermediates from reactions of O_2^- and H_2O_2 are known, and recent investigations have focused on determining whether PMN or MN make and release these other derivatives. The methods include measuring the production of ethylene from mixtures of phagocytes and thioethers, either B-methyl-proprionaldehyde (methional) or 2-keto-4-thiomethylbutyric acid (KMB) (28, 31, 32). This technique is based on the assumption that ·OH reacts to oxidize methional or KMB and thus releases ethylene (C_2H_4), $\cdot OH + HCOCH_2CH_2SCH_3 \rightarrow C_2H_4 + \frac{1}{2}(CH_3S)_2 + HCOOH$, or $\cdot OH + CH_3SCH_2CH_2COCOOH \rightarrow C_2H_4 + \frac{1}{2}(CH_3S)_2 + HCOOH + CO_2$, which can be measured by gas chromatography (4). By using this approach, it was found that stimulated PMN and MN produce C_2H_4 (27, 31, 32).

In contrast to PMN or MN, wide discrepancies are reported on the oxidative metabolism of alveolar macrophages (AM). Some investigators have suggested that AM either fail to produce O_2^- or H_2O_2 or do not release increased quantities during phagocytosis (5, 6, 30). In contrast, others have demonstrated O_2^- release, H_2O_2 or ·OH production which is enhanced during phagocytosis (7-9, 12). In addition, there are striking differences in oxidative metabolism of PMN or MN and AM in the same species (7, 8, 22). Because of these differences, we felt it important to determine and document whether human AM generate ·OH.

MATERIALS AND METHODS

Recovery and preparation of phagocytes. AM were obtained by bronchoscopic lavage of the unaffected subsegments of the lungs of patients undergoing diagnostic evaluations for localized pulmonary problems or healthy volunteers (11). Recovered cells were separated from lavage fluid by centrifugation, resuspended in Hanks balanced salt solution (HBSS), and counted. The purity of AM was greater than 90%. The concentration of PMN was <3%. When assessed by trypan blue exclusion, the viability of AM was ≧85%.

PMN were obtained from drug-free normal subjects and purified by using Ficoll-Hypaque differential density centrifugation. PMN were recovered, washed, and counted as previously described (23).

Preparation of serum and zymosan. Human serum from five normal subjects was obtained by venipuncture, allowed to clot, rimmed, recovered by centrifugation, pooled, and then frozen at −70°C for not more than 1 month (24). Zymosan A (50 mg, Sigma Chemical Co., St. Louis, Mo.) was washed with HBSS,

opsonized with pooled human serum at 37°C for 30 min, centrifuged at 700 × *g* for 5 min, and suspended in HBSS.

Measurement of hydroxyl radical production. Production of the hydroxyl radical was determined by measuring the amount of ethylene produced from mixtures of phagocytes and methional or KMB (28, 31, 32). Briefly, siliconized glass tubes were prepared by addition of 1 mM methional or KMB with either HBSS (1 ml, unstimulated samples) or HBSS with 15 mg of opsonized zymosan (1 ml, stimulated samples). Then AM or PMN, 2 × 10^7, in HBSS (2 ml) were added to the test vials. In some experiments, superoxide dismutase (600 U, Sigma), catalase (500 U, Sigma), sodium benzoate (20 mM, J. T. Baker, Phillipsburg, N.J.), or mannitol (20 mM, Sigma) were added before the addition of AM. Final reaction mixtures were 3 ml with an overlying gas space of 1.5 ml. After addition of AM or PMN, the vials were immediately stoppered, sealed, and incubated at 37°C for 20 min in a shaking water bath. At the end of the incubations, the reactions were stopped by placing the tubes on ice. The amount of ethylene produced was measured by injecting 1 ml of the overlying gas into the gas chromatograph (series 1400, Varian Instruments, Los Angeles, Calif.) equipped with a Chromosorb Carbosieve B60/40 column ⅛ inch by 6 foot [ca. 3.2 mm by 183 cm] (Supelco, Inc., Bellafontaine, Pa.). The injector, detector, column temperatures, and gas flow rates were adjusted so that the C_2H_4 peak occurred at 0.9 min after injection. The quantity of C_2H_4 3produced was calculated by comparing the areas under sample peaks to the areas from peaks produced by standard amounts of C_2H_4 in N_2 (Supelco).

Measurement of oxygen consumption. Oxygen consumption was determined by standard biological probe using a previously described method (11). The rate of oxygen taken up at 37°C was determined by measuring the slope for 5 min for 4 × 10^6 unstimulated or stimulated AM or PMN/ml in 8% serum. Maximal rates were reached rapidly and were linear throughout observation.

Measurement of superoxide anion. Release of superoxide anion by unstimulated or stimulated phagocytes was determined by measuring superoxide anion-dependent horse heart ferricytochrome *c* reduction spectrophotometrically (2, 13). Reaction mixtures contained 1 ml volume of buffered HBSS with 5 × 10^6 cells. In addition, some of the mixtures contained 150 U of superoxide dismutase. One of the paired mixtures was incubated for 20 min at 37°C; the other was kept on ice and used as a blank. At the completion of incubation, the reaction mixtures were kept at 4°C and centrifuged at 800 × *g*. The spectrums of the supernatants were measured at 550 nm and expressed as nanomoles of superoxide dismutase-inhibitable cytochrome *c* reduced ($\Delta E_{550\ nm}$ = 21 nm^{-1} cm^{-1}).

Measurement of total cellular protein. Cellular protein was determined by the Folin phenol reagent procedure of Lowry et al. on cell homogenates prepared by sonication and freeze-thawing (21).

Statistics. The results from individual determinations were averaged, and the statistical significance of the results was determined by non-paired *t* test analysis (26).

RESULTS

The results demonstrate that when stimulated, human AM generate C_2H_4 from methional or KMB (Table 1). C_2H_4 production by zymosan-stimulated AM (1,440 pmol/2 × 10^7 AM per 20 min) was significantly ($P < 0.05$) greater than unstimulated AM (180 pmol), HBSS, or HBSS with zymosan controls (120 or 140 pmol, respectively). Similarly with KMB, zymosan-stimulated AM made more C_2H_4 than unstimulated AM or controls. Additional experiments showed that increasing the number of stimulated AM progressively increased the amounts of C_2H_4 produced. In contrast, the levels of C_2H_4 production by unstimulated AM were not significantly increased over control levels. Thus, stimulated but not unstimulated AM made detectable C_2H_4.

To further elucidate the mechanisms responsible for the formation of C_2H_4, we determined

TABLE 1. *Effect of inhibitors on ethylene (C_2H_4) production by unstimulated or zymosan (Z)-treated human AM*

Test conditions	Concn of inhibitor	C_2H_4 produced (pmol/2 × 10^7 AM per 20 min)			
		with methional[a]	P[b]	with KMB	P
HBSS alone		120 ± 28 (10)	NS	38 ± 14 (9)	NS
AM + HBSS		180 ± 52 (5)	<0.05	46 ± 11 (4)	<0.05
AM + Z		1,440 ± 180 (5)		298 ± 36 (5)	
AM + SOD + Z	67 μg/ml	180 (3) 95%[c]		109 (3) 73%	
AM + catalase + Z	67 μg/ml	730 (3) 54%		130 (3) 65%	
AM + benzoate + Z	20 mM	300 (3) 86%		162 (3) 52%	
AM + mannitol + Z	20 mM	625 (2) 62%		185 (3) 43%	

[a] Mean ± standard error (number of determinations).

[b] NS, Not significant.

[c] Percent inhibition compared to AM + Z [1-(X − HBSS alone)/(AM + Z − HBSS alone)] × 100. Autoclaved SOD inhibited ethylene production from (AM + Z) + KMB by 11% autoclaved catalase inhibited ethylene production from (AM + Z) + KMB by 5%. See text for concentration of inhibitors.

the effect of a number of scavengers of O_2 intermediates on the production of C_2H_4 by stimulated AM (Table 1). Two agents which are believed to scavenge ·OH, sodium benzoate and mannitol, inhibited the production of C_2H_4 by stimulated AM. In addition, superoxide dismutase (SOD), which rapidly degrades O_2^- or catalase, which inactivates H_2O_2, both decreased the C_2H_4 production by stimulated AM. The actions of SOD and catalase appeared specific because heat inactivation of these enzymes abrogated their inhibitory effect. The latter findings support the role of O_2^- and H_2O_2 in the generation of C_2H_4 and suggest the existence of Haber-Weiss type reactions in stimulated AM (9):

$$O_2^- + H_2O_2 \text{ ----- } \cdot OH + OH^- + O_2$$

To further improve our understanding of AM metabolism, we compared O_2 uptake, O_2^- release, and C_2H_4 production by human AM and PMN (Table 2). We found that unstimulated AM used much more O_2 per cell (4×) than PMN, but that unstimulated AM and PMN made comparable amounts of O_2^- and C_2H_4. In contrast, stimulated AM and PMN used similar amounts of O_2, but PMN made more O_2 (2×) and more C_2H_4 (4×). Of note, the response of AM to stimulation was significantly ($P < 0.01$) less than that of PMN in regard to O_2 uptake, O_2^- release, and C_2H_4 production. When these results are compared on the basis of cell protein, the differences are even greater since AM have four times more protein per cell than PMN (41 ± 4.9 μg of protein per 10^5 AM; 10 ± 1.3 μg of protein per 10^5 PMN (mean ± standard error)). Although there are marked quantitative differences in C_2H_4 production by AM and PMN, the mechanism of C_2H_4 production is likely similar because scavengers of O_2 intermediates demonstrated similar effects on C_2H_4 production by stimulated AM or PMN (Table 3). Of note, while higher concentrations of catalase increase the degree of inhibition of ethylene production, the results must be interpreted with caution because of the nonspecific inhibition effected by inactivated catalase or bovine serum albumin at similar weight/volume concentrations. In summary, these findings confirm that although human AM and PMN are in many ways similar, there are marked quantitative differences in their generation of products of partially reduced oxygen.

TABLE 3. *Comparison of the effect of inhibitors on detection of C_2H_4 production from KMB by human AM or PMN stimulated by opsonized zymosan*

Agent (concn)	C_2H_4 production (% inhibition)	
	Stimulated AM	Stimulated PMN
SOD (67 μg/ml)	73 ± 8 (3)[a]	91 ± 1 (3)
SOD inactivated (67 μg/ml)	11 ± 6 (3)	4 ± 8 (3)
Catalase (67 μg/ml)	65 ± 7 (3)	42 ± 4 (4)
Catalase (330 μg/ml)		54 ± 9 (3)
Catalase inactivated (67 μg/ml)	5 ± 5 (3)	6 ± 7 (4)
Catalase inactivated (330 μg/ml)		34 ± 6 (3)
Albumin (67 μg/ml)	5 ± 6 (3)	3 ± 7 (3)
Albumin (330 μg/ml)		30 ± 5 (3)
Benzoate (20 mM)	52 ± 7 (3)	56 ± 8 (3)
Mannitol (20 mM)	43 ± 12 (3)	20 ± 10 (3)

[a] Mean ± standard error (number).

DISCUSSION

Prior investigations have shown that stimulated human PMN or MN, as well as rat phagocytes, make C_2H_4 from methional or KMB (8, 28, 31, 32). In the present investigation, we have documented that stimulated human AM also make ethylene from these reagents. In addition, the production of C_2H_4 by AM was inhibited by superoxide dismutase, catalase, mannitol, or sodium benzoate.

The present investigation has also revealed that there are significant differences in the amounts of C_2H_4 produced by human AM and PMN as well as differences in other metabolic activities of the two cell types, specifically O_2 uptake of O_2^- production. Although O_2 uptake

TABLE 2. *Comparison of the O_2 uptake, O_2^- generation, and C_2H_4 production of human AM and PMN*

Test conditions	O_2 Uptake[a] (μl/5 × 10^6 cells per h)	P[b]	O_2^- generation (nm/5 × 10^6 cells per 20 min)	P	C_2H_4 production (pmol/2 × 10^7 cells per 20 min)			
					Methional	P	KMB	P
Unstimulated AM	11 ± 1.1 (7)	<0.005	3.4 ± 0.9 (9)	NS	180 ± 52 (5)	NS	46 ± 11 (4)	NS
Unstimulated PMN	3.0 ± 1.0 (10)		2.6 ± 0.5 (6)		208 ± 61 (7)		107 ± 21 (9)	
Stimulated AM	19 ± 2.0 (7)	NS	8.7 ± 1.0 (9)	<0.01	1,440 ± 180 (5)	<0.01	298 ± 36 (5)	<0.01
Stimulated PMN	17 ± 2.1 (10)		15 ± 0.8 (6)		5,690 ± 98 (12)		985 ± 160 (10)	

[a] Mean ± standard error (number of determinations).
[b] NS, Not significant.

by stimulated AM or PMN is approximately equal, O_2^- release was about one half as great in stimulated AM as in PMN. This is because AM convert only a portion of the O_2 consumed during phagocytosis to reactive intermediates, whereas the majority is metabolized via the mitochondrial respiratory chain (12). These differences in oxidative metabolism may account in part for the decreased bactericidal activities of AM compared to PMN (27).

The significance of the production of ethylene from mixtures of phagocytes and thioethers is open to some question. Initial studies used methional as an indicator of ·OH production by phagocytes (28, 31). However, it was subsequently shown that methional spontaneously forms C_2H_4, especially with prolonged incubation (32). In the present study, C_2H_4 production from methional was measured, but the problem of spontaneous C_2H_4 generation was avoided by using short incubation times. In addition, KMB was also used as a substrate because it does not spontaneously form C_2H_4 (32).

The specificity of the production of ethylene from mixtures of phagocytes and thioethers also has been debated because of recent observations that catalase exerted little enzyme mediated effect on ethylene formation from reaction mixtures of PMN or PMN particulates and methional (29). It was suggested that oxidizing radicals generated by the reaction of O_2^- and compounds other than H_2O_2 might be responsible for generation of ethylene from methional or KMB. However, these results (29) contrast with the current investigation in which superoxide dismutase or catalase significantly inhibited C_2H_4 production by both AM and PMN, an effect which was reversed by heat inactivating the enzymes. From the current study, it would appear that both O_2^- and H_2O_2 are likely important participants in forming the oxidizing radical which reacts with the thioethers. The basis for these contrasting observations is not apparent. A possible important difference between the studies is that the initial catalase concentration used in the current investigations was about 25% that of the aforementioned study (29). As subsequently demonstrated, protein inhibits ethylene formation in a nonspecific manner. Therefore, at high concentrations of catalase, the specific effect of the enzyme may be obscured by the nonspecific effects of increased protein.

In summary, since a number of reactions are known by which O_2^- and H_2O_2 interact to produce ·OH, $^1O_2$, or other reactive O_2 byproducts, it seems likely that production of C_2H_4 is somehow representative of the production of these other O_2 derived species (not O_2^- or H_2O_2) by human AM. The exact nature of the reactive O_2 species produced awaits dissection by more sophisticated approaches. However, it is clear that human AM make C_2H_4 from methional or KMB, that these probably represent production of O_2 intermediates other than O_2^- or H_2O_2, and that the metabolic activities of AM and PMN are distinctly different.

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LITERATURE CITED

1. **Allen R. C., R. L. Stjernholm, and R. H. Steele.** 1972. Evidence for the generation of an electronic excitation state (s) in human polymorphonuclear leukocytes and its participation in bactericidal activity. Biochem. Biophys. Res. Commun. **47**:679–684.
2. **Babior, B. M., R. S. Kipnes, and J. T. Curnette.** 1973. Biologic defense mechanisms. The production by leukocytes of superoxide, a potential bactericidal agent. J. Clin. Invest. **52**:741–744.
3. **Baehner, R. L., and R. B. Johnston, Jr.** 1972. Monocyte function in children with neutropenia and chronic infections. Blood **40**:31–41.
4. **Beauchamp, C., and I. Fridovich.** 1970. A mechanism for the production of ethylene from methional: the generation of hydroxyl radical by xanthine oxidase. J. Biol. Chem. **245**:4641–4646.
5. **Biggar, W. D., and J. M. Sturgess.** 1978. Hydrogen peroxide release by rat alveolar macrophages: comparison with blood neutrophils. Infect. Immun. **19**:621–629.
6. **De Chatelet, L. R., D. Mullikin, and C. E. McCall.** 1975. The generation of superoxide anion by various types of phagocytes. J. Infect. Dis. **131**:443–446.
7. **Drath, D. B., and M. L. Karnovsky.** 1975. Superoxide production by phagocytic leukocytes. J. Exp. Med. **141**: 257–262.
8. **Drath, D. B., M. L. Karnovsky, and G. L. Huber.** 1979. Hydroxyl radical formation in phagocytic cells of the rat. J. Appl. Physiol. **46**:136–140.
9. **Gee, J. B. L., C. L. Vassalo, P. Bell, J. Kaskin, R. E. Basford, and J. B. Field.** 1970. Catalase dependent peroxidative metabolism in the alveolar macrophage during phagocytosis. J. Clin. Invest. **49**:1280–1287.
10. **Haber, F., and J. Weiss.** 1934. The catalytic decomposition of hydrogen peroxide by iron salts. Proc. R. Soc. Edinburgh Sect. A. **147**:332–351.
11. **Hoidal, J. R., G. D. Beall, F. L. Rasp, B. Holmes, J. G. White, and J. E. Repine.** 1978. Comparison of the metabolism of alveolar macrophages from humans, rats and rabbits: response to heat-killed bacteria or phorbol myristate acetate. J. Lab. Clin. Med. **92**:787–794.
12. **Hoidal, J. R., R. B. Fox, and J. E. Repine.** 1979. Defective oxidative metabolic responses *in vitro* of alveolar macrophages in chronic granulomatous disease. Am. Rev. Respir. Dis. **120**:613–619.
13. **Hoidal, J. R., J. E. Repine, G. D. Beall, F. L. Rasp, Jr., and J. G. White.** 1978. The effect of phorbol myristate acetate on the metabolism and ultrastructure

of human alveolar macrophages. Am. J. Pathol. **91**:469–483.

14. **Holmes, B. A., A. R. Page, and R. A. Good.** 1967. Studies of the metabolic activity of leukocytes from patients with a genetic abnormality of phagocytic function. J. Clin. Invest. **46**:1422–1432.
15. **Iyer, C. Y. N., M. F. Islam, and J. H. Quastel.** 1961. Biochemical aspects of phagocytosis. Nature (London) **192**:535–541.
16. **Johnston, R. B., Jr., B. B. Keele, H. P. Misra, J. E. Lehmeyer, L. S. Webb, R. L. Baehner, and K. U. Rajagopalan.** 1975. The role of superoxide anion generation in phagocytic bactericidal activity: studies with normal and chronic granulomatous disease leukocytes. J. Clin. Invest. **55**:1357–1372.
17. **Johnston, R. B., Jr., J. E. Lehmeyer, and L. A. Guthrie.** 1976. Generation of superoxide anion and chemiluminescence by human monocytes during phagocytosis and on contact with surface-bound immunoglobulin G. J. Exp. Med. **143**:1551–1556.
18. **Klebanoff, S. J.** 1975. Antimicrobial mechanisms in neutrophilic polymorphonuclear leukocytes. Semin. Hematol. **12**:117–142.
19. **Klebanoff, S. J., and R. A. Clark.** 1975. Hemolysis and iodination of erythrocyte components by myeloperoxidase-mediated system. Blood **45**:699–707.
20. **Krinsky, N. I.** 1974. Singlet excited oxygen as a mediator of the antibacterial action of leukocytes. Science **186**: 363–365.
21. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. **193**:265–275.
22. **Oren, R., A. E. Farnham, K. Saito, E. Milofsky, and M. L. Karnovsky.** 1963. Metabolic patterns in three types of phagocytosing cells. J. Cell. Biol. **17**:487.
23. **Repine, J. E., and C. C. Clawson.** 1978. Influence of surface proteins and separation techniques on neutrophil unstimulated and stimulated locomotion in vitro. Res. J. Reticuloendothel. Soc. **24**:217–226.
24. **Repine, J. E., J. G. White, C. C. Clawson, and B. M. Holmes.** 1974. The effect of phorbol myristate acetate on the metabolism and ultrastructure of neutrophils in chronic granulomatous disease. J. Clin. Invest. **54**:83–90.
25. **Salin, M. L., and J. M. McCord.** 1975. Free radicals and inflammation. J. Clin. Invest. **56**:1319–1323.
26. **Schorr, S. S.** 1968. Fundamentals of biostatistics, p. 137. G. P. Putnam & Sons, New York.
27. **Simmons, S. P., and M. L. Karnovsky.** 1973. Iodination ability of various leukocytes and their bactericidal activity. J. Exp. Med. **138**:44–63.
28. **Tauber, A. I., and B. M. Babior.** 1977. Evidence for hydroxyl radical production by human neutrophils. J. Clin. Invest. **60**:374–379.
29. **Tauber, A. I., T. G. Gabig, and B. M. Babior.** 1979. Evidence for production of oxidizing radicals by the particulate O_2^- forming system from human neutrophils. Blood **53**:666–675.
30. **Tsan, M. F.** 1977. Stimulation of the hexose monophosphate shunt independent of hydrogen peroxide and superoxide production by rabbit alveolar macrophages during phagocytosis. Blood **50**:935–945.
31. **Weiss, S. J., G. W. King, and A. F. LoBuglio.** 1977. Evidence for hydroxyl radical generation by human monocytes. J. Clin. Invest. **60**:370–373.
32. **Weiss, S. J., P. K. Rustagi, and A. F. LoBuglio.** 1978. Human granulocyte generation of hydroxyl radical. J. Exp. Med. **147**:316–323.