# HUMAN GRANULOCYTE GENERATION OF HYDROXYL RADICAL*

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As human leukocytes phagocytize, there is an associated burst of oxygen consumption (1), emission of light (chemiluminescence) (2), stimulation of the hexose monophosphate shunt (1), and the production of a variety of oxygen related toxic agents (3). These events appear to comprise an integral part of the anti-bacterial mechanism (4). Hypothesized mediators of the oxygen-dependent bactericidal event include superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), the hydroxyl radical ($OH^{\cdot}$), and singlet oxygen ($O_2^1$) (reviewed in reference 3). Recent observations have implicated an important role for the $OH^{\cdot}$, a potent oxidant, in both the bactericidal mechanism (5) and inflammation (6). In this report, we describe an assay system which provides evidence for the generation of $OH^{\cdot}$ by human granulocytes.

The assay system is based on the observation of Beauchamp and Fridovich (7) that the enzyme system xanthine-xanthine oxidase was capable of oxidizing methional ($\beta$-methylthiopropionaldehyde) with the generation of ethylene gas ($C_2H_4$) as an end product of this reaction. Ethylene generation was inhibited by $OH^{\cdot}$ scavengers and dependent on $O_2^{\cdot -}$ and $H_2O_2$. They suggested that $O_2^{\cdot -}$ and $H_2O_2$ interacted via the Haber-Weiss reaction to generate the $OH^{\cdot}$ with subsequent oxidation of methional. We have employed the modification of Heikkila et al. (8) substituting 2-keto-4-thiomethylbutyric acid (KMB)[1] for methional for the detection of $OH^{\cdot}$ as outlined below.

$$OH^{\cdot} + CH_3SCH_2CH_2COCOOH \rightarrow C_2H_4 + 1/2\ (CH_3S)_2 + HCOOH + CO_2$$

## Materials and Methods

*Preparation of Leukocytes.* Leukocytes were obtained by dextran sedimentation of blood from normal human volunteers. Granulocyte suspensions of a purity greater than 95% were obtained by Ficoll-Hypaque separation of the leukocyte-rich plasma and shock lysis of erythrocytes (9). Zymosan was opsonized by incubating 1 vol of zymosan (50 mg/ml) with 3 vol of fresh autologous serum for 30 min at 37°C. The particles were then washed and resuspended to a final concentration of 50 mg/ml as previously described (10).

*Determination of Ethylene Generation of PMNs.* Initial studies were carried out with methional as the source for ethylene generation as previously reported in our studies with monocytes (11). However, we discovered that this compound displayed auto-oxidative properties. Fig. 1 depicts the generation of $C_2H_4$ from methional or KMB in the absence of leukocytes or particles

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[1] *Abbreviations used in this paper:* DMF, dimethyl-furan; KMB, 2-keto-4-thiomethylbutyric acid; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocytes; SOD, superoxide dismutase.

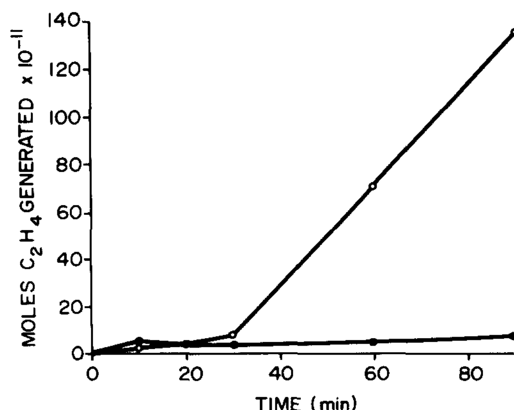



FIG. 1. Spontaneous ethylene generation by 1 mM methional (-O-O-O-O-) and 1 mM KMB (-●-●-●-●-) at 37°C in the absence of leukocytes or zymosan particles. The points represent the mean of duplicate observations.

at 37°C as a function of time. $C_2H_4$ generation from methional was minimal for the first 20 min with a subsequent rise in spontaneous $C_2H_4$ production. The possibility that the phagocytic event might trigger or accelerate this already spontaneous process suggested that utilization of KMB which is stable under these conditions would facilitate interpretation. Thus, $C_2H_4$ production was determined by incubating $2.5 \times 10^6$ PMNs in Hanks' balanced salt solution (pH 7.4) with 1 mM KMB in a final vol of 1.5 ml at 37°C. This concentration of KMB did not alter cell viability as judged by trypan blue exclusion and readily allowed detection of ethylene by phagocytic cells. The reaction took place in sealed, siliconized glass tubes with or without 5 mg of opsonized zymosan as the ingestable particle for periods of 30 or 60 min. This concentration of zymosan gave maximal $C_2H_4$ generation as well as maximal reduction of nitroblue tetrazolium, hexose monophosphate shunt, and chemiluminescence responses (unpublished observations). In some experiments, phorbol myristate acetate (PMA), a membrane stimulus not requiring the phagocytic process to generate oxygen radicals (12), was used in place of zymosan. Reactions were terminated by ice-bath temperatures and the addition of 1 mM *N*-ethyl-maleimide injected through the rubber stopper. Reduction of temperature and addition of *N*-ethyl-maleimide at 0 time completely inhibited $C_2H_4$ generation. 1-ml portions 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 at 110°, 150°, and 85°C, respectively. Standardization and quantitation of $C_2H_4$ with this system has been previously described (13).

*Ethylene Generation by the Xanthine-Xanthine Oxidase System.* The assay was performed according to the method of Beauchamp and Fridovich (7), with the exception of the substitution of KMB for methional. Briefly, a standard reaction mixture contained $1 \times 10^{-3}$ M KMB, $2 \times 10^{-4}$ M xanthine, $1 \times 10^{-4}$ M EDTA and $1.6 \times 10^{-8}$ M xanthine oxidase in a final vol of 1.0 ml buffered at pH 7.8 with 0.5 M potassium phosphate. Reactions were terminated after 15 min by ice bath temperatures.

*Special Reagents.* Catalase (type C-40) was obtained from Sigma Chemical Co., St. Louis, Mo. The catalase was freed of contaminating SOD by repeated washings over an XM-100A Diaflo ultrafiltration membrane from the Amicon Corp., Scientific Sys. Div., Lexington, Mass. Bovine superoxide dismutase (SOD) (3,000 U/mg) xanthine oxidase (125 U/ml), albumin, ascorbic acid, zymosan, xanthine, L-tryptophan, L-methionine, KMB, and methional were obtained from the Sigma Chemical Co. Sodium azide and potassium cyanide were obtained from Fisher Chemical Co., Fairlawn, N. J., *N*-ethylmaleimide from Kodak Chemical Co., and Hank's balanced salt solution from Grand Island Biological Co., Grand Island, N. Y. 2,5 dimethylfuran was purchased from the Aldrich Chemical Co., Milwaukee, Wis. PMA was a gift from Dr. R. B. Johnston (National Jewish Hospital and Research Center, Denver, Colo.).

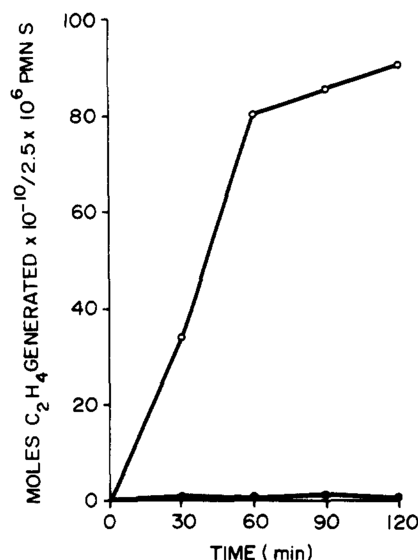



FIG. 2. Generation of ethylene from 1 mM KMB by granulocytes in the presence (-○-○-○-) or absence (-●-●-●-●-) of 5 mg/ml opsonized zymosan. Each point is a mean of duplicate observations.

## Results

The generation of $C_2H_4$ from granulocytes in contact with zymosan particles increased with time reaching a plateau at 60 min (Fig. 2). Little or no $C_2H_4$ appeared over this same time period in the absence of zymosan. The amount of $C_2H_4$ generated over a 60 min period bore a linear relationship to number of PMNs in the incubation mixture (Fig. 3) over a range in cells from 1-4 $\times$ $10^6$ cells. Thus, at a concentration of 2.5 $\times$ $10^6$ PMNs, the amount of particles or KMB were not rate limiting in terms of $C_2H_4$ generation.

Table I lists the effects of particulate and nonparticulate membrane pertubation on the $C_2H_4$ generation by human PMNs. The cells in the absence of specific stimuli had minimal $C_2H_4$ generation. A dramatic increment in $C_2H_4$ generation was seen with zymosan particles while PMA (1.0 $\mu$g/ml) produced a substantial increment over base line in the absence of an ingested particle.

Table II lists the results of an experiment to determine the dependence of $C_2H_4$ generation on $O_2^{\cdot -}$ and $H_2O_2$. SOD almost totally abolished $C_2H_4$ generation while catalase was only slightly less effective. The heat inactivated enzyme preparations had no inhibitory effect on $C_2H_4$ generation and albumin used as a protein solution control had only modest inhibitory activity. In seven experiments, SOD produced 94 $\pm$ 2% and catalase 74 $\pm$ 2% inhibition of $C_2H_4$ generation while in three experiments albumin produced 19 $\pm$ 3% inhibition.

We examined the effects of a variety of agents known to the $OH^{\cdot}$ scavengers in cell-free systems (Table III). Ethanol and benzoate produced a moderate degree of inhibition at concentrations used by other investigators to implicate a role of $OH^{\cdot}$ in granulocyte function (5). Methionine and tryptophan which have higher rate constants for $OH^{\cdot}$ (14) produced a more dramatic inhibition of $C_2H_4$ generation at relatively low concentrations. Ascorbate, a known $OH^{\cdot}$ and $O_2^{\cdot -}$

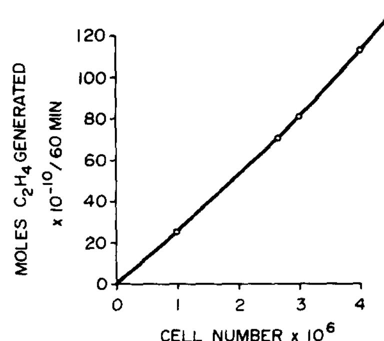



FIG. 3. Generation of ethylene by varying numbers of granulocytes in the presence of 5 mg/ml opsonized zymosan. Each point is a mean of duplicate observations.

TABLE I
*$C_2H_4$ Generation by Human Granulocytes*

| Additive | Experiments | $C_2H_4$ Generated* |
|---|---|---|
| None | 7 | 1.0 ± 1.1 |
| Zymosan, 5 mg | 7 | 64.0 ± 14.8 |
| PMA,‡ 1 μg/ml | 3 | 25.2 ± 7.2 |

* Mean × $10^{-10}$ mol ± 1 SD/2.5 × $10^6$ cells/60 min.
‡ Phorbol myristate acetate.

TABLE II
*Effect of SOD and Catalase on $C_2H_4$ Generation by Zymosan Stimulated Granulocytes*

| Additive | $C_2H_4$ Generated* | Inhibition |
|---|---|---|
| | | % |
| None | 20.1 | – |
| SOD (H.I.),‡ 10 μg/ml | 1.2 | 94 |
| Catalase (H.I.),‡ 250 μg/ml | 5.3 | 74 |
| Albumin, 250 μg/ml | 16.5 | 18 |
| SOD (H.I.),‡ 10 μg/ml | 22.1 | – |
| Catalase (H.I.),‡ 250 μg/ml | 21.0 | – |

* Expressed as moles of $C_2H_4$ × $10^{-10}$/2.5 × $10^6$ PMNs/30 min in the presence of 5 mg zymosan (mean of duplicate observations).
‡ Enzymes were heat inactivated by autoclaving.

scavenger (14, 15), also produced almost complete inhibition of $C_2H_4$ generation. Since tryptophan is also known to scavenge $O_2^1$ (16), we did additional studies comparing its effects on $C_2H_4$ generation with dimethyl-furan (DMF), a potent $O_2^1$ scavenger (17). In the cell-free xanthine-xanthine oxidase system, tryptophan (1 mM) produced 60 ± 7% ($n$ = 3) inhibition of $C_2H_4$ generation while DMF (1 mM) had no inhibitory effect. This enzyme system has been shown to generate $O_2^1$ (18) and the lack of DMF effect would suggest that $O_2^1$ contributes little to $C_2H_4$ generation from KMB. Similarly, DMF produced no reduction in $C_2H_4$ generation by granulocytes in contact with zymosan particles.

TABLE III
*Effect of Various Inhibitors on $C_2H_4$ Generation by Zymosan Stimulated Granulocytes*

| Additive | Experiments | Inhibition* |
|---|---|---|
| | | % |
| Ethanol, 40 mM | 6 | 38 ± 3 |
| Benzoate, 20 mM | 6 | 33 ± 3 |
| Tryptophan, 1 mM | 6 | 96 ± 1 |
| Methionine, 1 mM | 4 | 66 ± 4 |
| Ascorbate, 1 mM | 5 | 95 ± 1 |
| Cyanide, 1 mM | 5 | 82 ± 11 |
| Azide, 0.1 mM | 7 | 92 ± 4 |

* Mean ± 1 SD inhibition.

Since the myeloperoxidase-$H_2O_2$-halide system has been demonstrated to play a role in iodination, chemiluminescence and possibly the bactericidal event (19, 20), we examined the effect of chemical inhibition of myeloperoxidase on $C_2H_4$ generation. Azide and cyanide, potent inhibitors of this enzyme (19), dramatically inhibited $C_2H_4$ generation (Table III) by granulocytes. In contrast, these agents did not significantly impair $C_2H_4$ production by the xanthine-xanthine oxidase system. These concentrations of axide and cyanide do not inhibit phagocytosis (1, 21) or superoxide generation (22) by granulocytes.

## Discussion

We have demonstrated that granulocytes are capable of oxidizing KMB to $C_2H_4$ during phagocytosis or specific membrane perturbation. This $C_2H_4$ generation was significantly depressed by catalase and SOD. Since catalase reduces available $H_2O_2$ without affecting $O_2^{\cdot -}$ and SOD reduces $O_2^{\cdot -}$ while increasing available $H_2O_2$, we have interpreted these inhibitory data to mean that the $C_2H_4$ generated reflects a product of $H_2O_2$ and $O_2^{\cdot -}$ interaction. The $OH^{\cdot}$ radical or a $OH^{\cdot}$-dependent material would seem the likely candidate and this postulate is reinforced by the inhibition of $C_2H_4$ generation by $OH^{\cdot}$ scavengers. The interpretation of data with individual molecules having $OH^{\cdot}$ scavenger activity should be done with care since each of these agents probably has multiple effects on cell metabolism. However, the inhibition seen with five structurally unrelated scavengers in this assay suggests that the $C_2H_4$ generation is due to $OH^{\cdot}$. The varying degrees of inhibition by $OH^{\cdot}$ scavengers undoubtedly reflects their individual rate constants of interaction with $OH^{\cdot}$ as well as their ability to gain access to sites of radical generation in this cellular system. This latter characteristic of scavengers is largely unknown. The lack of effect of dimethylfuran on $C_2H_4$ generation by the cell free enzyme system as well as the granulocyte system is evidence against $O_2^1$ playing an important role in $C_2H_4$ generation.

We have previously shown that blood monocytes, a second phagocytic leukocyte with bactericidal and inflammatory functions, have the capacity to generate $C_2H_4$ from methional with similar dependence on both $H_2O_2$ and $O_2^-$ (11). Tauber and Babior, in a preliminary report, found $C_2H_4$ generation from methional by human granulocytes ingesting zymosan (23). However, in contrast

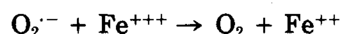
to our studies, they were unable to resolve the role of $H_2O_2$ since there was considerable inhibition of $C_2H_4$ production by heat inactivated catalase and albumin. Although their observations may be related to differing techniques, we feel that the autooxidative properties of methional probably indicates that KMB is a superior reagent for assaying $OH^\cdot$ generation in cellular systems.

The biochemical pathways for generation of $OH^\cdot$ in phagocytic leukocytes have not been established. One proposed mechanism involves the Haber-Weiss reaction (24) shown below.

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

An alternative reaction sequence involving iron chelates has been proposed by Fong et al. (25) as shown below:

$$H_2O_2 + Fe^{++} \rightarrow OH^\cdot + OH^- + Fe^{+++}$$



$$O_2^{\cdot-} + Fe^{+++} \rightarrow O_2 + Fe^{++}$$

The $H_2O_2$ is required for $OH^\cdot$ generation through a Fenton's reagent type reaction while $O_2^{\cdot-}$ is needed for regeneration of the ferrous form of the chelate. Either of these reactions for generation of $OH^\cdot$ would result in observations made with the $C_2H_4$ assay described in this report, i.e. dependence on $O_2^{\cdot-}$ and $H_2O_2$ as well as inhibition by $OH^\cdot$ scavengers.

However, the unexpected observation that both azide and cyanide inhibit cellular generation of $C_2H_4$ while the cell-free enzyme system is not adversely effected, complicates the interpretation of the data. Both these agents have the capacity to act as $OH^\cdot$ scavengers ($K_{CN^-}$ $4.5 \times 10^9$ $M^{-1}$ $S^{-1}$; $K_{N^-3} = 1.1 \times 10^{10}$ $M^{-1}$ $S^{-1}$) (14). Their failure to inhibit $C_2H_4$ generation in the xanthine-xanthine oxidase system could be due to the formation of cyanide or azide radicals (26) which are capable of oxidizing KMB in the cell-free system. In the more complex cellular system, redox agents may be present which are capable of reducing the cyanide or azide radical and, therefore, preventing oxidation of KMB to $C_2H_4$. Alternatively, the inhibition of $C_2H_4$ generation by cyanide and azide may indicate a role for myeloperoxidase in $OH^\cdot$ generation in granulocytes. Several investigators have demonstrated radical production by peroxidase systems (27–29). Yang described the ability of peroxidase to catalyze the conversion of either methional or KMB to $C_2H_4$ (30, 31). In this system, peroxidase served as a source of free radicals which catalyzed a radical chain propagation that led to the postulated generation of the $OH^\cdot$. In addition, peroxidase may form an oxygen adduct, oxyperoxidase, whose subsequent decay is capable of generating oxygen radicals (32). The role of peroxidase in $OH^\cdot$ generation will require further studies including investigation of granulocytes derived from donors who have a genetic deficiency in myeloperoxidase activity (21).

Prior studies have implicated $OH^\cdot$ in the biochemical armamentarium of the granulocyte with a role to play in inflammation and bactericidal activity (5, 6). The evidence for $OH^\cdot$ in these studies was based on the ability of $OH^\cdot$ scavengers to impair bactericidal activity (5) and granulocyte cell death (6). This study utilizes a more direct chemical assay of $C_2H_4$ generation from KMB which appears to reflect $OH^\cdot$ generation by human granulocytes. Use of this

assay may allow new insight into the biochemistry of oxygen radicals in cellular systems. Preliminary observations on the ability of cyanide and azide to inhibit $C_2H_4$ generation may indicate a role of myeloperoxidase in $OH^{\cdot}$ generation by human PMN's.

## Summary

Human granulocytes were capable of oxidizing 2-keto-4 thiomethylbutyric acid to ethylene during phagocytosis or membrane perturbation. The reaction required hydrogen peroxide and superoxide and in addition was inhibited by various hydroxyl radical ($OH^{\cdot}$) scavengers. These observations represent direct evidence for the generation of $OH^{\cdot}$ by human granulocytes. Further, inhibition of ethylene generation by azide and cyanide suggests that $OH^{\cdot}$ generation in granulocytes may be linked to myeloperoxidase.

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