Generation of Chemiluminescence by a Particulate Fraction Isolated from Human Neutrophils

ANALYSIS OF MOLECULAR EVENTS

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ABSTRACT A particulate fraction isolated from human neutrophils by homogenization, then centrifugation at 27,000 *g*, was demonstrated to generate chemiluminescence. This luminescence required the addition of reduced pyridine nucleotide and was very low in fractions from resting normal cells. Stimulation of neutrophils with opsonized zymosan, phorbol myristate acetate, or ionophore A23187 resulted in marked enhancement of the chemiluminescence measured in subsequently isolated particulate fractions. Stimulation did not boost the luminescence produced by fractions from cells of patients with chronic granulomatous disease. The chemiluminescence of particulate fractions from stimulated neutrophils was linear with increasing protein concentration, had a pH optimum of 7.0, and was higher with NADPH as substrate than with NADH. These results confirm previous studies suggesting that the enzyme system responsible for the respiratory burst in neutrophils is present in this fraction. The particulate fraction was used to examine the nature and origin of neutrophil luminescence by investigating the effect on this phenomenon of certain chemical and enzymatic scavengers of oxygen metabolites. Results suggest that the energy responsible for the luminescence of particulate fractions and, presumably, the intact cell, is derived from more than one oxygen species and that luminescence is a product of the interaction of these species and excitable substrates within the cell.

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INTRODUCTION

Phagocytosis by polymorphonuclear leukocytes (PMN)[1] elicits a burst of oxidative metabolism that is intimately involved in the killing of most microorganisms (1, 2). PMN from patients with chronic granulomatous disease, which cannot kill most bacteria and fungi, do not undergo this "respiratory burst" (2). Oxygen metabolites proposed to participate in this bactericidal activity include superoxide anion (O_2^-) (3), hydrogen peroxide (2), hydroxyl radical (·OH) (4–6) and singlet oxygen (7, 8). In addition, phagocytizing neutrophils from normal subjects (7) but not from patients with chronic granulomatous disease (9) generate a burst of luminescence that can be quantitated in a scintillation spectrometer. Singlet oxygen ($^1O_2$) was originally thought to be responsible for this luminescence, but analysis of the spectrum of light emitted by intact cells has indicated that the luminescence cannot be attributed to $^1O_2$ alone (10, 11). Previous attempts to identify the luminescing species have used certain scavengers and inhibitors; however, many of these compounds also affect other aspects of the phagocytic process in whole cells, such as binding and ingestion. We (12–14) and others (15–17) have shown NADPH-dependent oxidase activity and O_2^- production in a cell-free system isolated from human neutrophils. We now report that this system produces chemiluminescence that has characteristics like those of intact cells. By using the cell-free system to obviate the effects of organic scavengers on the function of whole cells, we were able to study the nature

[1] *Abbreviations used in this paper*: PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocytes; $^1O_2$, singlet oxygen; O_2^-, superoxide anion; ·OH, hydroxyl radical.

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and some of the subcellular requirements of the luminescence by neutrophils.

METHODS

Preparation of particulate fractions. Neutrophils were isolated from heparinized venous blood by dextran sedimentation (4), with final preparations of ≅85% PMN. In some instances, purer preparations (95–99%) were obtained by Ficoll-Hypaque centrifugation (Ficoll-Paque, Pharmacia Fine Chemicals Inc., Piscataway, N. J. (4) Comparative studies indicated that particulate fractions isolated from dextran-sedimented PMN had identical activity to fractions from the more purified preparations. Zymosan (ICN Nutritional Biochemicals, Cleveland, Ohio) opsonized with fresh serum (12) and phorbol myristate acetate (PMA) (Consolidated Midland Corp., Brewster, N. Y.) (13) were prepared as previously described. The ionophore A23187, generously donated by Dr. Robert L. Hamill (Eli Lilly & Co., Indianapolis, Ind.), was dissolved in 95% ethanol before use.

Isolated neutrophil preparations at 1.5×10^6/ml in either Dulbecco's phosphate-buffered saline or phosphate-buffered saline that contained 1.0 mM Ca^{++} and 1.25 mM Mg^{++} were incubated for 3 min at 37°C with either opsonized zymosan (11 mg/ml), PMA (0.1 μg/ml), A23187 (1 μM), or buffer alone. An equal volume of 0.68 M sucrose was added and samples were homogenized for 5 min (13). After a low speed (500 *g*) centrifugation, supernates were spun at 27,000 *g* (13). In some instances, sonication was substituted for the 5-min homogenization with no effect on final activity. Samples were sonicated on ice with a Branson Sonifier, model W185 (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) at a setting of 60 W in 30-s intervals for a total sonication time of 1–2 min. Final pellets were resuspended in 0.34 M sucrose to a protein concentration of 1–2 mg/ml (18) and stored at −20°C. This resuspended pellet is termed the particulate fraction. Stored fractions were stable over a period of at least 4–5 mo.

Chemiluminescence assays. Chemiluminescence was measured in the dark with a Beckman LS-100, ambient-temperature scintillation counter (Beckman Instruments Inc., Fullerton, Calif.) (4). The standard assay mixture contained the following in a 2.0-ml volume: 0.1 M potassium-phosphate buffer, pH 7.4, 0.2 mM NADPH, and 0.3–0.5 mg of particulate fraction. Assay components were added to dark-adapted, glass 20-ml scintillation vials, mixed, and placed immediately in the counter. Repetitive 0.2-min counts were recorded for ≅10 min. A peak of activity was routinely obtained between 1 and 2 min with particulate fractions from cells treated with opsonized zymosan. Data are reported as the peak counts per minute per milligram of protein corrected for background activity obtained with buffer alone. In some instances the following were included in the assay at concentrations indicated in the text: superoxide dismutase (Truett Laboratories, Dallas, Tex.); 1,4 - diazabicyclo[2.2.2]octane, 2,5 - dimethylfuran (Aldrich Chemical Co., Inc., Milwaukee, Wis.); 2,5-diphenylfuran (Eastman Kodak Co., Rochester, N. Y.); sodium formate (Mallinckrodt Inc., St. Louis, Mo.); 95% ethanol (U. S. Industrial Chemical Co., Div. of National Distillers and Chemical Corp., New York); sodium azide (Fisher Scientific Co., Fair Lawn, N. J.); catalase, bovine serum albumin, lysozyme, mannitol, sodium benzoate, L-tryptophan, L-histidine, hydroquinone or sodium ascorbate, all from Sigma Chemical Co., St. Louis, Mo.

An enzymatic superoxide anion-generating system was used to test the effects of various inhibitors and scavengers on chemiluminescence initiated by oxygen radicals. The assay is based on the procedure of Hodgson and Fridovich (19) and consisted of the following materials in a 2.0-ml volume: 0.2 M sodium-carbonate buffer, pH 10.0, containing 0.1 mM EDTA; 0.6 U xanthine oxidase, grade 1 (Sigma Chemical Co.); 0.1 M acetaldehyde (Eastman Kodak Co.) distilled before use; and the compound to be tested or its solvent. The luminescence emitted by this system was previously shown to be caused by the carbonate radical generated by the interaction of reactive oxygen species with carbonate buffer (19). Peak chemiluminescence obtained between 15 and 20 min, with background caused by buffer alone subtracted, was used to compare results.

Additional experiments explored the capacity of neutrophil particulate fractions to luminesce in the presence of oxygen radicals generated by either the xanthine-xanthine oxidase reaction or sodium superoxide, NaO_2 (Alfa Div., Ventron Corp., Danvers, Mass.). Xanthine-xanthine oxidase alone generates only low levels of luminescence, as a result of the absence of an excitable substrate such as carbonate, or perhaps the interaction of xanthine with oxygen radicals (19). The assay medium for the xanthine oxidase system consisted of 0.1 M potassium phosphate buffer, pH 7.4, 0.25 mM xanthine (Sigma Chemical Co.), 0.02 U xanthine oxidase, and 0.3–0.5 mg particulate fraction from either resting or phagocytizing cells, in a total volume of 2.0 ml. NaO_2 was prepared as a saturated solution, 5.5 mg/ml, in dimethylsulfoxide. The assay mixture consisted of 0.1 M potassium phosphate buffer, pH 7.4; 10 μl NaO_2; and 0.3–0.5 mg particulate fraction; in a total volume of 2.0 ml. Peak activity in each system was noted at the initial count, and background activity of buffer alone was subtracted.

NADPH oxidase activity was measured by a modification of the procedure of Babior et al. (17). The reduction of cytochrome *c* at 550 nm by O_2^- was followed on a Cary 219 double-beam recording spectrophotometer (Varian Associates, Instrument Div., Palo Alto, Calif.). The assay mix contained 0.05 M potassium-phosphate buffer, pH 7.0; 1.0 mM Na azide; 0.1 mM EDTA; 0.1 mM flavine adenine dinucleotide; 0.08 mM cytochrome *c*; 0.1 mM NADPH; and 0.2–0.5 mg/ml particulate fraction protein and was placed in both sample and reference cuvettes. In addition, the reference cuvette contained 50 μg/ml superoxide dismutase. Azide had no effect on activity. Initial slopes were used for calculations.

RESULTS

Particulate fractions isolated from phagocytizing neutrophils were found to generate chemiluminescence in the presence of NADPH. The typical pattern obtained is illustrated in Fig. 1. The NADPH-dependent luminescence of fractions from cells exposed to opsonized zymosan rose rapidly to a peak of ≅40,000–50,000 cpm/mg in 1–2 min and then declined. If NADPH were omitted or fractions from unstimulated cells were used, little activity was present.

A summary of peak activity in particulate fractions from resting and stimulated cells is given in Table I. Fractions from cells that had phagocytized opsonized zymosan had substantially greater activity than that found in fractions from unstimulated cells. The soluble stimuli, PMA and ionophore A23187, were also capable of activating PMN, in that fractions from these cells exhibited luminescence well above resting levels. With PMA as stimulus the time that activity peaked was the same as observed with opsonized zymosan (Fig. 1).

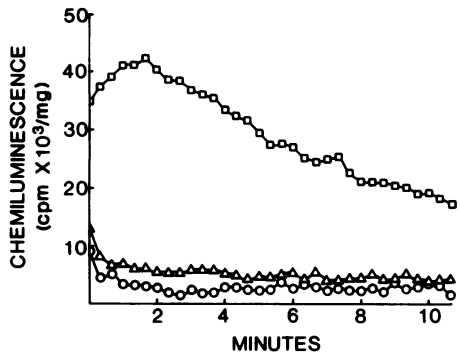

FIGURE 1 Chemiluminescence exhibited by particulate fractions from neutrophils, plotted as a function of time. Results are shown with fractions from cells of a single donor and are representative of those obtained with preparations from all normal individuals. The extent of luminescence is expressed as counts per minute per milligram of protein used. □, Particulate fraction from cells that had phagocytized opsonized zymosan, NADPH added; △, particulate fraction from cells that had phagocytized opsonized zymosan, no NADPH added; ○, particulate fraction from resting cells, NADPH added.

Peak of activity with A23187 was slightly delayed, occurring at 3–4 min. In parallel studies, A23187 also effectively stimulated NADPH oxidase activity in particulate fractions, as described previously for PMA and opsonized zymosan (13). With 0.2 mM NADPH as substrate, the nanomoles of $NADP^+$ produced per minute per milligram was as follows: resting, 0.05±0.03 (3); A23187-treated, 1.05±0.11 (3) [mean±SEM (*n*)].

In contrast to normal cells, no increase over resting levels of chemiluminescence was apparent in fractions from stimulated cells of five patients with chronic granulomatous disease (Table I). Cells from these patients had previously been shown to have defective NADPH oxidase activity (14).

TABLE I
Chemiluminescence of Particulate Fractions from Resting and Stimulated Neutrophils of Normal Subjects and Patients with Chronic Granulomatous Disease

Source of fraction	Stimulating agent	Chemiluminescence*
		cpm/mg
Normal cells	None	7,313±753 (38)
	Opsonized zymosan	47,626±2,422 (69)
	PMA	33,217±8,878 (14)
	Ionophore (A23187)	22,556±3,223 (2)
Patient cells‡	None	12,705±3,705 (2)
	Opsonized zymosan	12,310±2,851 (6)

* Values represent peak counts per minute per milligram of fraction used and are expressed as mean±SEM. The number of experiments performed is shown in parentheses.
‡ Fractions from cells of five patients with chronic granulomatous disease were studied under phagocytizing conditions (one patient was studied twice). Cells from two of these patients were studied under resting conditions.

Peak chemiluminescence in fractions from phagocytizing cells was linear with respect to protein concentration as shown in Fig. 2A. On the other hand, increased amounts of protein had little effect on the low level of activity observed in particulate fractions from resting cells, indicating the lack of an efficient luminescence-generating system in these fractions. A pH optimum of 7.0 was observed with fractions from phagocytizing cells (Fig. 2B). The chemiluminescence of intact cells stimulated by opsonized zymosan at pH 6.5, 7.0, 7.4, and 8.0 was also optimal at pH 7.0 ($n = 3$).

The dependence of activity on reduced pyridine nucleotide was further examined. As shown in Fig. 3, activity was greater in the presence of NADPH than NADH, especially at low substrate concentrations. When NADH was used as substrate, the time at which activity peaked and the shape of the curve obtained with time were the same as seen with NADPH (Fig. 1), suggesting that the same process was occurring in the use of both nucleotides.

The origin of neutrophil chemiluminescence was explored with particulate fractions from phagocytizing cells by examining the effect of enzymatic and chemical scavengers. Activity in the presence of the enzymes superoxide dismutase and catalase is summarized in Table II. Both enzymes lowered activity, although complete inhibition was not obtained. Simultaneous addition of the two enzymes did not further increase inhibition over that seen with superoxide dismutase alone, suggesting that both enzymes acted at the same point. In control experiments, neither enzyme inhibited significantly when inactivated by autoclaving, and 100 μg each of two other proteins, bovine serum albumin and lysozyme, had no effect on activity.

Table III summarizes the effect of various chemical scavengers on the chemiluminescence of particulate fractions. Three different scavengers of singlet oxygen, azide (20), hydroquinone (21), and 1,4-diazabicyclo-[2.2.2]octane (22), were found to inhibit peak activity. Azide was particularly potent, causing 66% inhibition at 5 μM. Diphenylfuran, dimethylfuran, and histidine, also reported to be $^1O_2$ scavengers (23–26), either stimulated chemiluminescence or had no effect on activity. We examined the action of these agents on the chemiluminescence of the xanthine oxidase-acetaldehyde system, which is mediated by oxygen radicals (19). All three agents stimulated luminescence in this system, making it impossible to interpret their effects in the particulate fraction system in regard to $^1O_2$.

The inhibition by both superoxide dismutase and catalase suggested that hydroxyl radical, formed via the Haber-Weiss reactions (27), could be involved in

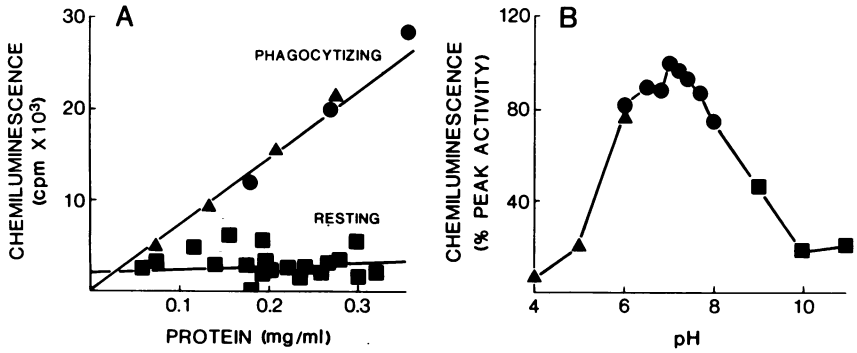

FIGURE 2(A) Effect of increasing concentrations of protein on the chemiluminescence of particulate fractions from resting PMN and from PMN that had phagocytized opsonized zymosan. Fractions from two separate stimulated cell preparations were used (●, ▲). Each resting value (■) represents a separate cell preparation. The resting plot was made by linear regression analysis. Correlation coefficients were 0.997 for the data from fractions of phagocytizing cells and 0.173 for the data from fractions of resting cells. (B) Effect of pH on chemiluminescence of particulate fractions from cells that had phagocytized opsonized zymosan. The results shown represent the mean of two experiments with different particulate fractions. The buffers used at 0.1 M final concentration were: ▲, citrate-phosphate; ●, potassium phosphate; and ■, glycine-sodium hydroxide. If a mixture of half potassium phosphate and half glycine-hydroxide was used at pH 8.0 and 9.0, respectively, to overlap buffers, the results obtained at these pH values were the same as those shown in the figure.

the generation of chemiluminescence. Several ·OH scavengers (19, 28, 29) were tested for inhibitory effect. The addition of ethanol resulted in a slight inhibition. Tryptophan and formate were more effective, although at relatively high concentrations. Neither mannitol nor benzoate had any effect on activity of the particulate fraction or of the control xanthine oxidase-acetaldehyde system. Ascorbic acid, believed to be a general scavenger of oxygen radicals (30), was also a potent inhibitor.

We explored the possibility that the inhibitory effect of the various "scavengers" might be caused by inhibition of generation of oxygen radicals through a direct effect on the oxidase enzyme. Of the inhibitory compounds shown in Tables II and III, none except ethanol had any significant effect on NADPH oxidase activity in the particulate fraction measured by the superoxide-mediated reduction of cytochrome *c*. Because ascorbate and superoxide dismutase remove O_2^-, they could not be tested in the cytochrome assay. They also have been shown to inhibit the oxidation of NADPH by the particulate fraction (13, 31), which has been attributed to removal of the direct oxidative effects of O_2^- (31). Ethanol produced a 48% inhibition of NADPH-dependent O_2^- generation, thus its slight effect on chemiluminescence is difficult to interpret.

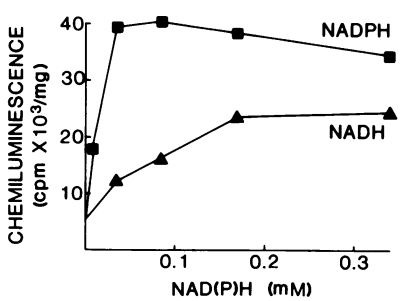

FIGURE 3 Effect of reduced pyridine nucleotide concentration on the chemiluminescence of particulate fractions from cells that had ingested opsonized zymosan. Experiment shown is representative of three performed. The differences between NADH and NADPH are significant ($P < 0.05$) at the 0.034, 0.085, and 0.017 mM concentrations using the one-tailed paired *t* test.

We also investigated the possibility that excitable components of the particulate fraction could be acting to amplify the chemiluminescence generated by oxygen metabolites. Results are summarized in Table IV. The xanthine-xanthine oxidase system, known to produce O_2^- (32), generated only a small amount of luminescence. However, in the presence of the particulate fraction, the system produced high levels of chemiluminescence. Activity was primarily dependent on the presence of xanthine oxidase and the particulate fraction, as the elimination of xanthine from the system only lowered activity by ≅30%. This suggested that the particulate fraction contains a substrate that can be used by xanthine oxidase. However, increasing the concentration of xanthine in the system, already 100 times the reported K_m (33), by 10-fold in the presence or absence of the particulate fraction did not increase the luminescence generated. (This result is not surprising

TABLE II

Effect of Superoxide Dismutase and Catalase on the Chemiluminescence of Particulate Fractions from Stimulated Cells

Addition	Activity*
μg/ml	%
Superoxide dismutase,	
12.5	61±3 (3)
50	48±3 (5)
100	36±8 (3)
Catalase (A),‡	
50	67±6 (3)
100	55±5 (3)
Catalase (B),‡	
20	34±12 (4)
Superoxide dismutase and catalase (A),‡	
50 each	36±4 (3)
Autoclaved superoxide dismutase,	
50	86±2 (4)
Autoclaved catalase (B),‡	
20	124±15 (3)
Bovine serum albumin,	
50	97±5 (3)
Lysozyme,	
50	99±9 (3)

* Peak activity in the presence of the added material compared to peak activity obtained with buffer in matched experiments. Values are expressed as mean±SEM. The number of experiments is given in parenthesis. Particulate fractions were obtained from cells that had ingested opsonized zymosan.

‡ Two preparations of catalase were tested. Preparation A was used as available from the commercial source. Preparation B was further purified by gel chromatography with Bio-Gel A-0.5 (Bio-Rad Laboratories, Richmond, Calif.).

in view of reported inhibition of xanthine oxidase by excess substrate [34]). Also dialyzed particulate fraction was as effective as fresh material in boosting luminescence. Thus, it appeared highly unlikely that luminescence enhancement by the particulate fraction in the complete system was caused by addition of substrate for the xanthine oxidase.

The presence of excitable substrates in the particulate fraction was further supported by examination of a chemical source of O_2^-, NaO_2 (sodium superoxide). Alone, NaO_2 was a poor emitter of chemiluminescence. However, addition of particulate fraction produced a 10-fold enhancement of this luminescence. Fractions isolated from resting cells were as effective in generating luminescence as were fractions from phagocytizing cells in both systems, indicating that excitable components are present in equivalent concentrations in fractions from both resting and stimulated cells.

A similar phenomenon was observed with active particulate fraction as the source of oxygen radicals. If increasing amounts of resting particulate fraction was added to a fixed amount of phagocytizing particulate fraction in the presence of NADPH, a linear increase in activity was observed (data not shown). However, this activity was not as high as that observed in Fig. 2A, when the source of protein was solely particulate fraction from phagocytizing cells. Thus, the activity observed in active particulate fraction is dependent on both the amount of O_2^--generating enzyme and the amount of excitable substrate available.

TABLE III

Effect of Chemical Scavengers of Oxygen Metabolites on the Chemiluminescence of Particulate Fractions from Phagocytizing Cells

Addition	Activity*
	%
Azide,	
0.05 μM	80±8 (3)
5 μM	34±7 (3)
DABCO,	
1 mM	92±3 (3)
10 mM	41±1 (3)
Hydroquinone,	
1 μM	59±10 (3)
10 μM	0 (3)
Diphenylfuran,	
1 mM‡	104±6 (4)
Dimethylfuran,	
10 mM	185±27 (3)
Histidine,	
10 mM	118±3 (3)
Ethanol,	
2%	87±5 (3)
4%	85±5 (3)
Tryptophan,	
20 mM	40±9 (3)
Benzoate,	
10 mM	92±8 (3)
Mannitol,	
10 mM	95±3 (3)
Formate,	
10 mM	33±3 (3)
100 mM	0 (3)
Ascorbate,	
10 μM	79±6 (4)
0.1 mM	9±9 (3)

DABCO, 1,4-diazabicyclo [2.2.2]octane.

* Peak activity in the presence of the added material compared to peak activity obtained with solvent. Values are expressed as mean±SEM; the number of experiments is given in parentheses. Particulate fractions were isolated from cells that had phagocytized opsonized zymosan.

‡ The limit of solubility of this compound.

TABLE IV
Enhancement by the Particulate Fraction of the Chemiluminescence Produced by Two Sources of Superoxide Anion

Condition	Activity*	
	NaO_2	Xanthine oxidase
	%	
Complete system	100 (8)	100 (7)
Omit xanthine	—	71.3±4.1 (7)
Omit xanthine oxidase	—	17.6±3.2 (7)
Omit NaO_2	8.6±1.0 (8)	—
Omit particulate fraction	9.1±1.0 (8)	5.5±1.3 (7)
Omit xanthine oxidase and particulate fraction	—	0 (4)

* Expressed as the mean±SEM of the peak activity of the complete system (xanthine + xanthine oxidase + particulate fraction; NaO_2 + particulate fraction). The mean counts per minute at peak with 0.2 mg/ml fraction protein was 34,205 ±4,544 for the NaO_2 system and 42,455±11,737 for the xanthine oxidase system. The number of experiments is shown in parentheses. In each system four experiments were performed with fractions isolated from cells that had ingested opsonized zymosan, and either three or four with fractions from unstimulated cells.

DISCUSSION

It is clear from the experiments reported here that the particulate fraction isolated by centrifugation at 27,000 *g* has the capacity to generate chemiluminescence. This chemiluminescence closely resembles that of the intact cell (4, 9, 11, 35) and parallels previous measurements of O_2^- production (17) and NAD(P)H-oxidase activity (12–16) in this fraction. The activity is found in fractions from normal PMN that have been activated by phagocytosis or soluble stimuli of the respiratory burst, but not in fractions from resting cells. It is also deficient in fractions from PMN of patients with chronic granulomatous disease. This activity requires the presence of reduced pyridine nucleotide, NADPH being more effective than NADH. The optimal pH of 7.0 coincides with the reported pH optimum for O_2^- production by this fraction (17). These data provide substantial confirmation for the concept that the particulate fraction contains the enzyme system responsible for the respiratory burst in the intact cell.

It appeared valid then to use this fraction to analyze the molecular basis of the luminescence generated by PMN. The particulate fraction offers significant advantages over the intact cell in that studies can be performed without concern for the integrity of the cell or the functions of binding and ingestion. Results with various oxygen-radical scavengers suggested that several reactive species of oxygen are involved in the generation of light. Interpretations regarding particular species must be made with caution because the action of many of these inhibitors may not be entirely specific. However, it seems clear that O_2^- and H_2O_2 are participating in the luminescence, based on the inhibition by superoxide dismutase and catalase. Three known scavengers of $^1O_2$ (azide, 1,4-diazabicyclo[2.2.2]octane, and hydroquinone) were inhibitory. Three which did not inhibit (dimethylfuran, diphenylfuran, and histidine) were shown to accentuate the light emission initiated by oxygen radicals in the control xanthine oxidase-acetaldehyde system. It is impossible to reach definite conclusions from these results regarding the role of $^1O_2$ in chemiluminescence. The agents that inhibited luminescence could have done so through inhibition of a radical other than $^1O_2$ or, perhaps through inactivation of excitable substrate(s). Those agents that did not inhibit chemiluminescence could have scavenged $^1O_2$ while also serving as substrates capable of excitation by $^1O_2$ or by other oxygen species.

The same questions can be raised regarding the possible role of ·OH in chemiluminescence. Although superoxide dismutase and catalase inhibited luminescence, the only effective ·OH scavenger was tryptophan, and it also has been reported to quench $^1O_2$ (25, 26). Neither mannitol nor benzoate had an effect, whereas formate, although inhibitory, is also a known trap for H_2O_2 (36). However, because ·OH is so highly reactive, it is possible that components of the particulate fraction may successfully compete with added scavengers for any generated ·OH.

Ascorbate has been shown to interact with O_2^- (30) and to inhibit the chemiluminescence of intact PMN (37), the production of ·OH by PMN (6), and the activity of NADPH oxidase in the isolated particulate fraction (31). All of these effects of ascorbate, as well as the inhibition of the luminescence generated by the particulate fractions, could be explained by removal of O_2^-.

Superoxide and H_2O_2 are believed to interact in the Haber-Weiss reactions, catalyzed by heavy metals, to form ·OH (27, 38). Singlet oxygen has been proposed to be formed either directly in the reaction (39) or indirectly as the product of ·OH and O_2^- (40). The lack of consistent inhibition by ·OH quenchers in our system would favor the direct formation of $^1O_2$ in the Haber-Weiss reaction if, indeed, $^1O_2$ plays any part in this phenomenon.

Another approach to detection of $^1O_2$ depends on the formation of a specific product, cis-dibenzoylethylene, by the interaction of diphenylfuran and $^1O_2$ (41, 42). However the specificity of this reaction has recently been questioned (43), and our attempts to find product formation by the particulate fraction system in the presence of diphenylfuran have been unsuccessful.

Although oxygen radicals appear to participate in the chemiluminescence, other components of the particulate fraction may serve as luminescing species. Neither the xanthine-xanthine oxidase O_2^--generating system nor NaO_2 emitted significant luminescence. The addition of particulate fraction (in the absence of NADPH) resulted in a substantial burst of activity, indicating that excitable substrates in the fraction were participating in the light emission. Moreover, these substrates were found in equivalent concentrations in fractions from both resting and stimulated cells. The results with the NaO_2 system indicate that the sequence of events resulting in chemiluminescence by the particulate fraction can be initiated by the generation of O_2^- alone.

Taken together, our results and previous observations concerning the spectrum of light emission by phagocytosing PMN (10, 11) suggest that the generation of chemiluminescence by neutrophils consists of several steps. First, O_2^- is generated by the respiratory burst enzyme, which is active in stimulated but not in resting cells. The O_2^- may then interact directly with excitable substrates (44) or be converted to other oxygen metabolites which react with cell constituents to amplify the effect, resulting in the final luminescence observed.

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