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Research Article

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The Origin of the Chemiluminescence of Phagocytosing Granulocytes

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ABSTRACT Granulocytes engaged in the phagocytosis of opsonized zymosan emit light by a process that is inhibited by superoxide dismutase and catalase. In the present report it is shown that light emission is the result of reactions between certain unspecified constituents of the ingested particles and some or all of the oxidizing agents (H_2O_2, O_2^-, and possibly the hydroxyl radical and singlet oxygen) produced by the activated cells. This conclusion is based on a study of light emission by both activated cells and an artificial O_2^--generating system containing xanthine oxidase and purine. With these two systems, light production required the presence of both zymosan and oxidizing agents, suggesting that the oxidation of particle components is necessary for luminescence to occur. The characteristics of the emission spectrum as well as the finding that granulocytes activated by a nonparticulate agent (F^-) fail to luminesce show that light emission by the relaxation of singlet oxygen to the ground state does not contribute in a major way to the chemiluminescence of phagocytosing granulocytes; whether singlet oxygen contributes to chemiluminescence in other ways cannot be decided from the data available. Inasmuch as the oxidation of constituents of ingested particles is an important bacterial killing mechanism in the granulocyte, chemiluminescence may be viewed as a manifestation of the microbicidal activity of the cell.

INTRODUCTION

The bactericidal capacity of the polymorphonuclear leukocyte is, in part, dependent upon certain oxidative reactions that take place with the phagocytosis of the microorganisms (2–4). The first of these reactions involves the one electron reduction of oxygen by a pyridine nucleotide to form the highly reactive compound, superoxide (5–9). Subsequent reactions give rise to hydrogen peroxide (10–12) and, possibly, other reactive compounds.

One of the compounds postulated to be formed by activated granulocytes is singlet oxygen, an exceptionally reactive, electronically excited form of oxygen. The evidence for the production of this compound by the granulocyte is the observation by Allen et al. in 1972 (13) and later by others (14, 15) that neutrophils generate light after phagocytosis of opsonized bacteria, latex particles, or zymosan. Light is known to be produced in systems containing singlet oxygen, arising either from singlet oxygen itself as it relaxes to the ground state or from electronically excited compounds formed in secondary reactions between singlet oxygen and other molecular species (e.g., species with carbon-carbon double bonds, which react with singlet oxygen to form dioxetanes that in turn cleave to form aldehydes and ketones with the emission of light (16)). In further studies, Allen et al., (17), using opsonized bacteria, demonstrated a 30% reduction in light emission with the addition of superoxide dismutase, an enzyme that catalyzes the dismutation of superoxide to hydrogen peroxide and oxygen (18–20). That portion of light emission that was inhibited by dismutase was attributed to singlet oxygen generated from superoxide, while the remaining luminescence was attributed to unspecified hydrogen peroxide-requiring reactions which are unaffected by the further addition of dismutase. Webb et al. (14), using opsonized zymosan, noted a 70% inhibition of chemiluminescence with superoxide dismutase, supporting the role of superoxide in the light emitting process. They also used catalase, which decomposes hydrogen peroxide to oxygen and water, and benzoate, a scavenger of hy-

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droxyl radicals, to investigate the possibility that these compounds contributed to the remaining luminescence. Since hydrogen peroxide is a product of the dismutation of superoxide, the slight but consistent decrease in light emission observed with catalase suggested a role for hydrogen peroxide in light generation. The addition of benzoate also resulted in a small but consistent decrease in luminescence, suggesting that hydroxyl radicals also participated in light emission. The mechanism proposed for the production of hydroxyl radicals by granulocytes involved the reaction between superoxide and hydrogen peroxide first described by Haber and Weiss (21). Recent work by Klebanoff and Rosen has shown that azide, which inhibits myeloperoxidase, also interferes with chemiluminescence (22).

The present studies were designed to investigate the source of the luminescence of activated granulocytes, and, in particular, to establish the extent to which the relaxation of singlet oxygen to its ground state is responsible for this light. Further studies were also carried out to explore the role of superoxide and hydrogen peroxide in the chemiluminescence phenomenon.

METHODS

Materials. Human granulocytes were prepared as previously described (6). After isolation, the cells were suspended in either Hanks' buffered saline solution (HBSS; Grand Island Biological Co., Grand Island, N. Y.)[1] or, if they were to be exposed to NaF, in a phosphate-buffered saline solution (PBS) of the following composition: NaCl 0.14 M, KCl 2.7 mM, Na_2HPO_2 12 mM, KH_2PO_4 1.5 mM, $CaCl_2$ 0.9 mM, and $MgCl_2$ 0.49 mM. The cells were counted in a hemacytometer and the suspension diluted to a concentration of 10^7 cells/ml.

Zymosan was obtained from either Sigma Chemical Co. (St. Louis, Mo.) or ICN Pharmaceuticals Inc., Life Sciences Group (Cleveland, Ohio). To opsonize the particles, a suspension was prepared containing 50 mg zymosan in 3 ml fresh human serum and 1 ml buffer (either HBSS or PBS, depending on the experiment in which the zymosan was to be used). The suspension was incubated for 30 min at 37°C, then spun at 1,500 rpm for 10 min in Vari-Hi-Speed centrifuge (Precision Scientific Co., Chicago, Ill.) at ambient temperature. The supernate was removed and the particles were washed in 4 ml buffer. After repeat centrifugation, the supernate was again discarded, and the zymosan was resuspended in buffer at a concentration of 12.5 mg/ml.

Superoxide dismutase was obtained from Truett Laboratories (Dallas, Tex.) and dissolved in either HBSS or PBS at a concentration of 3 mg/ml. Bovine liver catalase (Sigma Chemical Co.) was dissolved fresh daily in distilled water at 15 mg/ml. Milk xanthine (Grade I) and purine were purchased from Sigma Chemical Co. Before use, xanthine oxidase (3 ml) was dialyzed against HBSS for 15 hours at 4°C. The protein concentration was measured after dialysis. A 1.0-M solution of purine in HBSS was prepared fresh daily. Horse heart ferricytochrome *c* (Sigma grade VI) was dissolved in distilled water to a concentration of 1.5 mM. Other reagents were the best grade commercially available and were used without further purification.

Measurement of light emission. Light emission was measured in a Packard Tri-carb model 3320 liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.) in the off-coincidence mode, with the tritium window setting as described by Allen and coworkers (13, 17, 23). The temperature inside the counter was maintained at 4°C. Unless otherwise specified, the constituents of the reaction mixures were placed in siliconized 20-ml glass scintillation vials which had been kept in the dark for several hours before use.

With the exception of the emission spectroscopy and NaF studies, experiments were performed in the following fashion. Four reaction mixtures were studied simultaneously. At time = 0, either cells or xanthine oxidase, depending on the experiment, were added to the appropriate reaction mixtures. The first vial was placed in the counter while the other samples were continuously agitated on an Ames rocker (Ames Co., Div. of Miles Lab, Elkhart, Ind.) at ambient temperature. After being counted for 30 s the first vial was immediately removed from the counter and returned to its place on the rocker. After each of the four samples had been counted in turn, the sequence was begun again and repeated continuously throughout the duration of the incubation period. Despite the fact that the temperature inside the scintillation counter was 4°C, the temperatures of the reaction mixtures remained constant during the course of the experiment. Mixtures were prepared and experiments were performed in dim light to minimize spurious light emission from the vials themselves.

Emission spectroscopy. Because of the low light emission levels, the spectra were obtained with a primitive broad band spectrometer of very high sensitivity which employed the scintillation counter operating in the off-coincidence mode as the detector and a set of flexible, colored plastic filters as the dispersive element. The filters used were as follows (Kodak Wratten no., Eastman Kodak Co., Rochester, N. Y. [cut-on wavelength[2] in nanometers]): 2A (418), 3 (462), 4 (471) 9 (508), 16 (535), 21 (555), 23A (579), 29 (618), and 92 (636). Each filter was cut to line the inner wall of a scintillation vial, the bottom of which was covered with a disk of aluminum foil applied with glue. The spectral transmittance curves of the filters were measured on a McPherson 700 series double-beam spectrometer (McPherson Instrument Co., Acton, Mass.) which had been calibrated against National Bureau of Standards transmission standards. The filters used were of the "sharp cut" variety with transmission at high wavelengths. The transition between high and low tranmission is abrupt, typically occurring over a 10–20-nm range.

For spectroscopy, the sample was placed in a thin-walled glass test tube cut to fit into a scintillation vial without leaking when the capped vial was inverted. The double container served to thermostat the reaction mixture at ambient temperature during the experiment. Measurements of luminescence were made as follows. A sample was placed in one vial, light emission was measured for 30 s in the liquid scintillation counter, the sample was placed in another vial and inverted once or twice, and the measure-

[1] *Abbreviations used in this paper:* λ_i, cut-on wavelength of filter *i*; λ_j, cut-on wavelength of filter *j*; HBSS, Hanks' buffered saline solution; *k*, unknown instrumental constant; *L*, normalized luminescence through a given filter; $P(\lambda)$, relative sensitivity of the detector; PBS, phosphate-buffered saline; $S(\lambda)$, luminescence spectrum; $T(\lambda)$, transmission spectrum of a given filter.

[2] The wavelength at which the transmittance was 50% of maximum for the filter.

ment was repeated. The process was continued until all the filtered vials had been used. Because of the decay of the luminescence, normalization of the light levels was necessary to obtain a spectrum. This was accomplished by alternating readings of samples contained in filtered vials with readings from the same sample in an unfiltered vial. The normalized luminescence was taken to be the ratio of counts through a given filter divided by the average of the counts obtained for the two unfiltered measurements bracketing the filtered reading. Several measurements were taken for each filter. Experiments in which the filters were used in order of ascending cut-on wavelength gave normalized luminescences in agreement with runs in which the filters were used in descending order. This indicated that although the intensity of light emission declined with time, the spectrum did not change.

The normalized luminescence through a given filter (L_i) is given by

$$L_i = k \int T_i(\lambda) S(\lambda) P(\lambda) d\lambda, \quad (1)$$

where $T_i(\lambda)$ is the transmission spectrum of filter i, $S(\lambda)$ is the luminescence spectrum, $P(\lambda)$ is the relative sensitivity of the detector (in this case, an RCA-8575 photomultiplier tube, RCA Corp., New York; the sensitivity curve was supplied by the manufacturer), and k is an unknown instrumental constant which depends on the absolute quantum efficiency of the photomultiplier, the efficiency of the collection optics, etc. When the true value of k is included in the calculation, L_i represents absolute luminescence, expressed as photons per unit time. For the present study, however, it was only necessary to calculate the relative luminescence, in which calculation k appears only as an arbitrary scaling constant of unknown magnitude whose value does not affect the results. Comparison of the normalized relative luminescence through any two of the filters provides information about the luminescence spectrum in the wavelength region spanned by the difference spectrum of two filters. Because of the sharp cut-on of the filters, it is reasonable to calculate an average spectral intensity $S(\lambda_i,\lambda_j)$ for $\lambda_i<\lambda<\lambda_j$ where λ_i and λ_j are the cut-on wavelengths of filters i and j. By employing an average photomultiplier sensitivity $P(\lambda_i,\lambda_j)$ in the same interval, it is possible to calculate a histogram of the luminescence:

$$S(\lambda_i,\lambda_j) = (L_i - L_j)/kP(\lambda_i,\lambda_j) \int (T_i[\lambda] - T_j[\lambda])d\lambda. \quad (2)$$

L_i and L_j are measured, $P(\lambda_i,\lambda_j)$ is determined from the photomultiplier sensitivity curve, and $(T_i[\lambda] - T_j[\lambda])d\lambda$ is obtained by integrating the difference spectrum of the two filters. Taking luminescence differences cancels the dark counts due to the photomultiplier. This is particularly useful in the extreme red regions ($\lambda > 600$ nm) where the insensitivity of the photomultiplier results in signals comparable with the dark noise.

The spectral transmission curves of the filters are not step functions. Because of this, the wavelength intervals represented by the histogram are somewhat approximate. In the present experiments the spectrum was partitioned into intervals proportional to the areas obtained from integration of the filter difference spectra. This procedure proved consistent with the actual cut-on wavelengths of the filters employed.

Determination of O_2^- production by granulocytes. Granulocyte O_2^- production was determined as described by Curnutte et al. (24), except for differences in the composition of the reaction mixtures and the duration of the incubations.

Protein determinations. Protein concentration was determined by the Lowry method (25).

RESULTS

Luminescence of granulocytes. With a reaction mixture allowing for the phagocytosis of opsonized zymosan by granulocytes we obtained light emission similar to that reported by previous workers (13–15, 17). Light emission with time demonstrated a peak at 10–20 min, followed by a slow decline (Fig. 1). The light emitted by the suspension of cells plus zymosan exceeded by far the light generated by buffer alone or by a suspension of either cells or zymosan (data not shown).

As reported before (14), chemiluminescence was inhibited by both superoxide dismutase and catalase (Fig. 1). When superoxide dismutase was present in a reaction mixture containing cells plus zymosan, the initial peak of luminescence was eliminated, but the curve of light emission continued to rise, meeting that of the control at approximately 60 min. To rule out the possibility that this rise in chemiluminescence was due to inactivation of the dimutase by H_2O_2 (26), experiments were conducted in which reactions initiated either in the absence or presence of superoxide dismutase were supplemented at times varying from 20–50 min with 0.1 ml of dismutase. There was no inhibition of luminescence by dismutase added after 20 min as compared to a control suspension supplemented with a similar volume of buffer.

Incubation of granulocytes with catalase allowed for a higher early peak than with superoxide dismutase, but at 60 min light emission was only 40% of control.

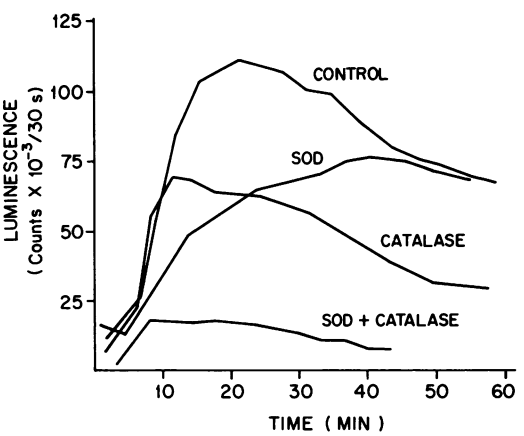

FIGURE 1 The effect of catalase and superoxide dismutase (SOD) on the chemiluminescence of phagocytosing granulocytes. The reaction mixture is the same as described in Table I. Where indicated, superoxide dismutase (0.6 mg), catalase (3 mg), or both were present. The incubations were begun with cells. Chemiluminescence was followed with time as described in Methods. Halving the concentration of either dismutase or catalase did not alter chemiluminescence in reaction mixtures containing these enzymes.

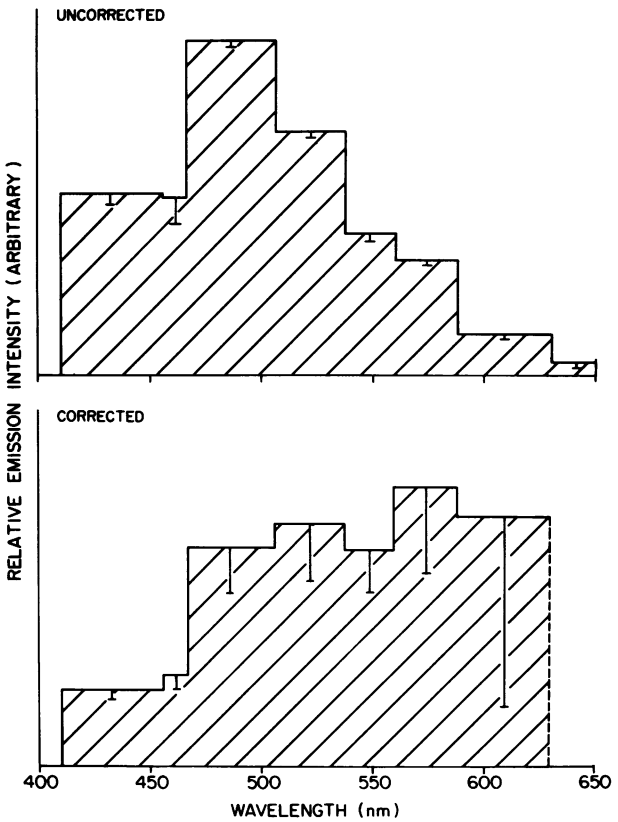

FIGURE 2 The spectrum of the light emitted by phagocytosing granulocytes. The reaction mixture contained 10^7 cells and 5 mg opsonized zymosan in 4.5 ml HBSS. The spectrum was obtained as described in Methods. (Top) The uncorrected spectrum. The area under each line segment is proportional to the relative luminescence intensity before correcting for the wavelength sensitivity of the photomultiplier. The relative luminescence intensity is proportional to the number of counts detected in the window between adjacent filters. It was calculated from Eq. 2 by taking $P(\lambda_i,\lambda_j)$ to be constant. Error bars indicate -1 SD. (Bottom) The corrected spectrum. The spectrum was calculated from Eq. 2 as described in Methods, determining $P(\lambda_i,\lambda_j)$ for each interval from the sensitivity curve of the photomultiplier tube. The significance of the error bars is discussed in the text.

When both dismutase and catalase were used, light emission throughout the incubation was reduced to less than 20% of control. Thus, it appears that superoxide is involved in most of the early light emission, while hydrogen peroxide is involved in the luminescence later in the reaction.

The spectrum of the light emitted by phagocytosing granulocytes. The luminescence spectrum of phagocytosing granulocytes is presented in Fig. 2. The spectrum obtained before correcting for the wavelength dependence of the photomultiplier sensitivity is shown in the top panel, while the bottom panel shows the corrected spectrum. The errors in the top spectrum are largely due to statistical fluctuations, while the errors in the corrected spectrum are almost entirely the result of variations in the photomultiplier response over the rather large bandwidths of the histogram. Within a given bandwidth, the range in photomultiplier response determines the maximum errors in the corrected spectrum. To the extent that the actual luminescence spectrum is broad and unstructured, errors of this type will tend to cancel.

There is a steep fall off in the photomultiplier sensitivity for wavelengths greater than 600 nm. The uncertainties in the corrections to be applied are so large that the values for the longest wavelength interval studied have not been included in the corrected spectrum.

The corrected spectrum shows that the chemiluminescence from phagocytosing granulocytes has substantial intensity throughout the visible spectrum. The essentially "white light" nature of the spectrum provides little support for the suggestion that singlet oxygen per se is a major emitter in this system (13, 27, 28). In particular, we have not observed the distinctive singlet oxygen emissions at 480 and 580 nm. On the other hand, our photomultiplier is not sensitive to the more intense singlet oxygen emissions at 633, 704, 762, and 1,070 nm (27). If there is singlet oxygen emission in these samples, it is masked by luminescence from other emitters

TABLE I
Chemiluminescence by Zymosan Plus Xanthine Oxidase-Purine

Conditions	Light emission		
	Exp 1	Exp 2	Exp 3
	counts/30 s		
Complete mixture	51,400	39,700	22,200
Omit xanthine oxidase	6,100		
Omit purine	9,500	4,000	
Omit zymosan	4,400		
Complete mixture, boiled xanthine oxidase*		4,400	
Complete mixture plus superoxide dismutase			4,400
Complete mixture plus catalase			22,800
Complete mixture plus mannitol			23,000

The complete reaction mixture contained 95 μg crude dialyzed xanthine oxidase, 0.9 mmol purine and 5 mg opsonized zymosan in 4.95 ml HBSS, with omissions as noted. Where indicated, superoxide dismutase (0.3 mg), catalase (3.0 mg), or mannitol (0.05 M) was also present. The incubation was begun by the addition of enzyme, and luminescence was measured at 3 min as described in Methods.
* The supernate from a portion of enzyme that was heated for 1 min in boiling water.

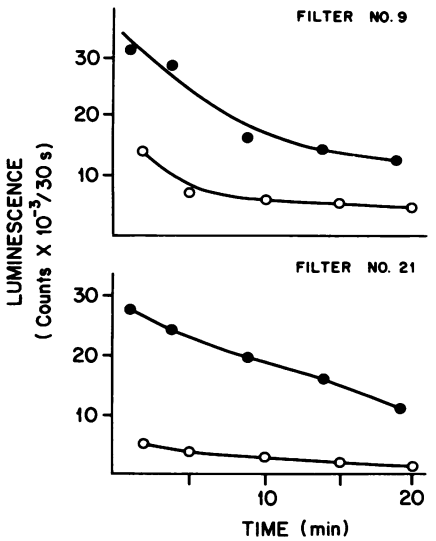

FIGURE 3 The spectrum of light emitted by the xanthine oxidase-purine-zymosan system. The reaction mixture was the same as described in Table II, except that for this experiment the quantity of xanthine oxidase was 170 μg. The mixture was placed in a test tube cut to fit inside a scintillation vial as described in Methods. The reaction vessel was placed alternately in an unfiltered vial (●) and a vial lined with the filter indicated (○), and readings taken at the times shown. Between readings, the reaction mixture was agitated by inversion at frequent intervals.

more easily demonstrated with our detection system. It should be noted that previous observations of chemiluminescence from phagocytosing granulocytes have also used modified liquid scintillation counters for detection (13, 14, 17). Those experiments, therefore, were performed with equipment similarly insensitive to the intense long wavelength singlet oxygen emissions.

Production of chemiluminescence by zymosan in the presence of the xanthine oxidase-purine system. A clue to the identity of the luminescing species was provided by the observation that in all experiments in which the luminescence of opsonized zymosan was compared to that of buffer alone, higher counts were seen with the zymosan (e.g., Table I). This observation, together with the work showing that superoxide and hydrogen peroxide were necessary for chemiluminescence, suggested that perhaps light emission occurs as the result of an interaction between one or both of the oxidizing species and zymosan. To test this hypothesis, we examined the ability of an artificial superoxide generating system composed of xanthine oxidase plus purine (29) to replace granulocytes as a source of chemiluminescence. The results (Table I) show that light was generated by a suspension of opsonized zymosan incubated with xanthine oxidase plus purine. With this system less light was emitted than with intact cells, and the rise and fall in light emission was more rapid (see below). Suspensions from which any of the reagents was omitted failed to luminesce, as did a suspension containing heat-denatured xanthine oxidase. Superoxide dismutase virtually abolished luminescence in this system, but catalase and mannitol (a scavenger of hydroxyl radicals) had little effect.

The rapid changes in the luminescence of this system precluded the measurement of a complete spectrum of the emitted light. It was possible, however, through the use of two cut-on filters, to obtain some information regarding this spectrum. The filters used in this experiment were Wratten no. 9, which transmitted 41% of the light produced by granulocytes plus zymosan, and Wratten no. 21, which transmitted 13% of this light (Fig. 2 uncorrected). Fig. 3 shows that similar proportions of the light generated by the xanthine oxidase-purine-zymosan system were transmitted by these filters. As with the intact cell, the spectrum of light emitted by this system appeared not to change with time. Thus, as far as could be determined under the present experimental conditions, the spectra resulting from incubating

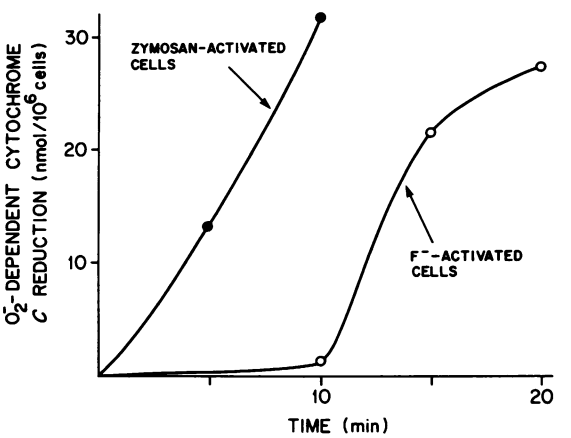

FIGURE 4 Activation of granulocyte O_2^- production by opsonized zymosan and by fluoride. For measuring O_2^- production by zymosan-activated neutrophils, the reaction mixtures contained 10^7 cells, 7.5 mg opsonized zymosan, 0.3 μmol cytochrome *c* and (where necessary) 60 μg superoxide dismutase in 4.0 ml PBS. Incubations were conducted at 37°C in a Dubnoff shaker. O_2^--dependent cytochrome *c* reduction was determined at 5 and 10 min as described in Methods. For measuring O_2^- production by F^--activated neutrophils, the reaction contained 10^7 cells, 0.3 μmol cytochrome *c* and (where necessary) 60 μg superoxide dismutase in 4.2 ml PBS containing 0.02 M NaF. F^--containing solutions were prepared immediately before use by dissolving the appropriate amount of NaF in PBS contained in a 50-ml plastic Falconware tube (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). In all experiments in which F^- was used, only plastic or siliconized glassware and pipettes were allowed to come into contact with the F^-. Incubations were conducted at 37°C in a Dubnoff shaker, and O_2^--dependent cytochrome *c* reduction was determined at 10, 15, and 20 min as described in Methods.

zymosan with either intact granulocytes or the artificial superoxide generating system were the same.

Luminescence of fluoride-activated granulocytes. The above evidence suggested the likelihood that chemiluminescence requires an interaction between granulocyte-generated superoxide and hydrogen peroxide on one hand, and zymosan on the other. This hypothesis predicts that light emission should not occur with granulocytes stimulated by a nonparticulate agent. Fluoride is such an agent, since it has been shown to be capable of stimulating the cellular oxidative burst (30, 31), resulting in the production of large quantities of superoxide (32). In Fig. 4 the activation of neutrophils by F^- is compared to that by zymosan. The rates of superoxide production by the stimulated cells are of the same order, although activation by F^- is associated with a significant lag not seen with zymosan.

In contrast to the similarity in the rates of superoxide production by cells activated by either agent, light emission rates differed greatly (Table II). While large amounts of light were generated by the particle-activated cells, chemiluminescence by F^--activated cells was negligible. This difference appeared to be a fundamental property of the system rather than a secondary effect due to quenching of light by F^-, since zymosan-activated cells introduced into a fluoride-containing buffer luminesced with the same intensity as did cells introduced into a similar buffer devoid of F^-. It thus appears that the production of superoxide and hydrogen peroxide by granulocytes is not sufficient for chemiluminescence to take place; particles must be present as well.

TABLE II

Comparison of Light Emission by Zymosan-Activated and Fluoride-Activated Granulocytes

Preparation	Light emission		
	Exp 1	Exp 2	Exp 3
	*counts/30 s**		
Fluoride-activated cells	100	100	2,300
Zymosan-activated cells	71,800	204,100	161,700
Diluted into F^--containing PBS	10,600	26,300	20,200
Zymosan-activated cells	63,900	184,400	147,600
Diluted into F^--free PBS	9,300	25,800	20,900

Three reaction mixtures were prepared, two containing granulocytes activated by zymosan and one containing cells activated by fluoride. For activation by zymosan, the reaction mixture contained 10^7 cells and 7.5 mg opsonized zymosan in 4.0 ml PBS. For activation by fluoride, reaction mixtures contained 10^7 cells in 4.0 ml PBS containing 0.02 M NaF (fluoride-containing solutions were prepared and handled as described in Fig. 4). Activation was accomplished by incubating the reaction mixtures in siliconized liquid scintillation vials at 37° in a Dubnoff metabolic shaker. The chemiluminescence of zymosan-activated cells was measured at 5 min, and that of fluoride-activated cells was measured at 15 min. Immediately after the measurement of light emission, a 1-ml portion of one of the zymosan-activated reaction measure was added to 1 ml of PBS containing 0.04 M NaF, while 1 ml of the other zymosan-activated mixture was added to 1 ml of PBS alone. In each case the diluent was contained in a siliconized liquid scintillation vial and was held at ambient temperature. The diluted mixtures were briefly agitated and light emission was again measured.

* Corrected by subtracting the mean dark count (value for the dark count was 2,400±100 SD [n = 25]).

DISCUSSION

Stimulated granulocytes generate several powerful oxidizing agents. H_2O_2 and O_2^- formation have been demonstrated directly by a number of experimental techniques (2–8, 10–12), while the production of hydroxyl radical, singlet oxygen or both have been inferred from several rather convincing but indirect observations (8, 17, 18, 28, 29, 33). H_2O_2 has been clearly shown by Klebanoff to participate in bacterial killing, (3, 34, 35), and there is some evidence that O_2^- may play such a role as well (8, 29, 36).

The present studies strongly suggest that chemiluminescence by phagocytosing granulocytes is the result of reactions between these oxidizing agents and certain constituents of the ingested particles (in the present case, opsonized zymosan). This conclusion is based on the finding that mixtures containing either neutrophils or the xanthine oxidase-purine system as a source of these oxidizing agents only emitted light in the presence of particles. Direct evidence for the involvement of the particles in light emission was provided by the findings that zymosan itself luminesced weakly and that its luminescence was greatly amplified in the presence of xanthine oxidase plus purine, a system known to generate the oxidizing agents listed above (i.e., H_2O_2, O_2^-, hydroxyl radical, and singlet oxygen). On the other hand, the oxidizing agents alone were not sufficient for light emission, because when they were generated either by xanthine oxidase plus purine in the absence of zymosan or by fluoride-stimulated neutrophils no luminesence was detected. Since oxidation of the constituents of ingested particles is an important killing mechanism in neutrophils, chemiluminescence can be viewed as a manifestation of the microbicidal activity of these cells.

Although light emission is clearly a concomitant of the oxidation of particle components, it is difficult to be precise about the reaction or reactions responsible for this phenomenon. Light emission is seen with many chemical oxidations, including the oxidations of simple aldehydes and ketones as well as of more complex compounds such as luminol (3-aminophthalhydrazide) and luciferin, the latter being the compound responsible for the luminescence of fireflies (37, 38). Other compounds of biological importance have also been implicated in chemiluminescence: the breakdown of organic peroxides in the presence of metalloporphyrins has been shown to be associated with light emission (38), and Vohaben and Steele have described a light-emitting system consisting of H_2O_2, riboflavin, and ascorbic acid, the emission spectrum of which is very similar to the spectrum obtained

in the present studies (39). With regard to the oxidizing agent, chemiluminescence is typically observed in peroxide-mediated oxidations (37), but the singlet oxygen-mediated cleavage of double bonds is also associated with light emission (see above), while the data reported in Table I suggests that O_2^--utilizing reactions can result in the emission of light as well. It seems reasonable to postulate that the light emitted by phagocytosing granulocytes represents a composite of many luminescent reactions involving the oxidation of a variety of particle constituents (e.g., flavins and unsaturated fatty acids) by any of the oxidizing agents liberated by the activated cells, reactions which, in some instances at least, are catalyzed by myeloperoxidase, as the data of Klebanoff and Rosen demonstrate (22).

In discussions of the chemiluminescence of phagocytosing granulocytes, the role of singlet oxygen has frequently been mentioned (13, 17, 28). It can be stated with some assurance that singlet oxygen itself is not a major emitter in this system. Direct evidence against this possibility is found in the spectrum of the emitted light, most of which appeared at wavelengths which did not include the singlet oxygen peaks. Further evidence is provided by the observation that cells stimulated by fluoride emitted no light. Since these cells release large quantities of O_2^- and H_2O_2, and since singlet oxygen is produced during reactions involving these two species (see below), we would have expected to see chemiluminescence regardless of the presence or absence of particles if singlet oxygen were the emitting species. We therefore conclude that singlet oxygen per se contributes little, if anything, to the luminescence of phagocytosing granulocytes.

This conclusion notwithstanding, we wish to emphasize that our study provides no information as to whether or not singlet oxygen is generated by phagocytosing granulocytes. The failure to detect chemiluminescence attributable to the relaxation of singlet to triplet (ground state) oxygen does not constitute evidence against its formation, since it can be consumed in chemical reactions, some of which yield other light-emitting species, and, in addition, can relax to the ground state by nonradiative processes in which its excess energy is liberated as heat, not light (40, 41). In view of the fact that singlet oxygen is a product of at least two reactions which could, in principle, take place in the phagocytosing granulocyte, namely the Haber-Weiss reaction (42):

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

and the reaction of H_2O_2 with the OCl^- produced by the action of myeloperoxidase (27, 43):

$$H_2O_2 + OCl^- \rightarrow H_2O + \text{singlet } O_2 + Cl^-,$$

and since O_2^-, H_2O_2, and OCl^- are produced by granulocytes, it seems not only possible, but even likely, that singlet oxygen is produced by these cells and participates in their antimicrobial function. To prove this, however, will require further investigation.

APPENDIX

The errors in Fig. 2 were determined according to standard treatments of error propagation (44). Starting with Eq. 2, the uncertainties in the histogram are given by:

$$\sigma S(\lambda_i,\lambda_j) = S\left\{\frac{(\sigma L_i)^2 + (\sigma L_j)^2}{(L_i - L_j)^2} + \frac{(\sigma P[\lambda_i,\lambda_j])^2}{(P[\lambda_i,\lambda_j])^2} + \frac{(\sigma A[i,j])^2}{(A[i,j])^2}\right\}^{\frac{1}{2}},$$

where $A(i,j) = (T_i[\lambda] - T_j[\lambda])d\lambda$.

σL_i, σL_j, and $\sigma A(i,j)$ are the standard deviations as determined from repeated measurements on these quantities. $\sigma P(\lambda_i,\lambda_j)$ are as explained in the text, maximum errors determined by the variation in photomultiplier response over the bandwidths of the histogram.

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