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Chemiluminescence Spectra of Human Myeloperoxidase and Polymorphonuclear Leukocytes

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The light emission spectra of myeloperoxidase-H_2O_2-Cl^- and phagocytizing polymorphonuclear leukocytes were estimated by a computer simulation technique by using light transmittance data from nine band-pass filters. No shift in the chemiluminescence spectrum of either system was observed during the course of the reactions, suggesting that the light-generating mechanisms remain constant after their initiation. Transmittances were virtually identical for both myeloperoxidase-H_2O_2-Cl^- and polymorphonuclear leukocyte reactions, suggesting that the light-generating mechanisms are identical and leading to the estimation of nearly identical spectra. Both spectra were broad, with maximum light emission near 570 nm.

Experiments by Allen and co-workers have demonstrated that light is emitted by both phagocytizing polymorphonuclear (PMN) leukocytes (2) and isolated myeloperoxidase (MPO) in the presence of H_2O_2 and Cl^- (1). The mechanisms of light production have remained unclear, although increased levels of several oxygen intermediates have been demonstrated in phagocytizing PMNs, including superoxide anion ($\cdot O_2^-$) (3) and H_2O_2. Allen et al. (2) first proposed that singlet oxygen ($^1O_2$) was also present, but only recently have Rosen and Klebanoff provided experimental evidence (Fed. Proc. 35:1391, 1976). The hydroxyl radical ($\cdot OH$) also has been postulated (10) to be another intermediate present in phagocytizing PMNs.

Of the intermediates listed above, only $^1O_2$ is in an electronically excited state as is required for chemiluminescence (CL). The others are theoretically capable of producing substances, such as $^1O_2$, which are electronically excited and therefore may be indirectly related to the light production.

In displaying CL, $^1O_2$ may decay to ground state O_2, releasing its energy as light, or it may react with other molecules, forming products that subsequently emit light. The CL spectrum of phagocytizing PMNs as shown by Cheson et al. (5) seems to rule out the direct decay of $^1O_2$ as the major source of CL. Although it is suspected that these light-emitting reactions are related to bactericidal mechanisms, proof is lacking. The present study demonstrates that the emission spectrum of a cell-free MPO-H_2O_2-Cl^- system is similar to that of phagocytizing PMNs.

MATERIALS AND METHODS

PMN preparation. Heparinized normal human blood was separated by Ficoll-Hypaque sedimentation (4) followed by dextran sedimentation for 45 min. The PMN-rich supernatant was washed and suspended in 0.9% saline. To lyse contaminating erythrocytes, 1 part of the cell suspension was mixed with 3 parts of distilled water for 20 s. The cells were returned to isotonicity by the addition of 1 part of 3.6% saline (6). This method yielded cell preparations of at least 98% PMNs. The PMNs were washed and suspended in phosphate-buffered saline (0.03 M phosphate, pH 7.3, containing 1 mg of glucose per ml) at a concentration of 5×10^6 to 7×10^6 cells per ml.

MPO preparation. Partially purified MPO was isolated from 7×10^7 normal human PMNs by using the weak acid extraction method (8). The activity was determined with *o*-dianisidine (7).

CL measurement. Light emission was measured in a scintillation spectrometer (Beckman model LS-250; photomultiplier tubes [PMTs]; RCA-4501/V3), with the refrigeration and coincidence circuits off, the amplification set to maximum, and a fully open discriminator window. Light emission was recorded as counts per minute. All light-generating reactions were performed in small (7.5-ml, 1.7-cm-diameter) glass scintillation vials, which had been placed within larger (22-ml, 2.7-cm-diameter) glass vials. A gelatin band-pass absorption filter (Edmund Scientific Co.), through which all emitted light had to pass before reaching either PMT, had been inserted into the space between the two vials. The light transmission characteristics of the nine filters used

were determined with a double-beam spectrophotometer (Beckman Acta-CV) (Fig. 1). In all experiments, another double vial that had no filter was included (unfiltered vial).

In all PMN experiments, 5 ml of the cell preparation was added to the inner vials and background counts were obtained. To initiate CL, opsonized zymosan (4 mg of zymosan incubated with 0.5 ml of normal human serum for 10 min at room temperature) was added. Then, 1-min CL measurements were executed sequentially on each of the 10 vials for over 1 h.

In the MPO experiments, background counts were determined on 2 ml of isolated MPO (1,750 U/ml in 0.05 M sodium acetate buffer, pH 3.5). CL was initiated by adding 0.5 ml of H_2O_2 (3% in 0.15 M NaCl). Light measurements were repeatedly and continuously made for 0.5-min intervals on each of the 10 vials for at least 6 min.

Data analysis. (i) Calculation of light transmittance. The time integral of the emitted light, corrected for background, was calculated for the reactants in each vial. For the unfiltered vial, this integral will be termed L_0; for each filtered vial, the integral will be designated L_i, where the subscript i is the filter number. For either system of reactants, the percent transmittance (T_i) through filter number i may be calculated as:

$$T_i = (L_i/L_0) \times 100 \quad (1)$$

(ii) Computer simulation. The light emitted (l_0) by the reactants in an unfiltered vial and received by the PMT is:

$$l_0 = \int_\lambda S(\lambda)P(\lambda)d\lambda \quad (2)$$

where $S(\lambda)$ is the emission spectrum of the reactants, $P(\lambda)$ is the quantum efficiency characteristic of the PMT, and λ is wavelength. The quantum efficiency of the PMT used in this study (RCA-4501/V3) was inadequate for reliable results when the wavelength of light was greater than approximately 620 nm.

Similarly, l_i is defined as the light emitted by the reactants in a vial containing filter number i and received by the PMT:

$$l_i = \int_\lambda S(\lambda)F_i(\lambda)P(\lambda)d\lambda \quad (3)$$

where $F_i(\lambda)$ is the transmittance spectrum of filter number i. Assuming the emission spectrum of the reactants remains unaltered with time, then if L_0 and L_i are calculated over the same time interval,

$$l_i/l_0 = L_i/L_0 \quad (4)$$

In the computer simulation, an estimated emission spectrum $\hat{S}(\lambda)$ was formed as will be described. The estimated luminosity was then computed as:

$$\hat{l}_0 = \int_\lambda \hat{S}(\lambda)P(\lambda)d\lambda \quad (5)$$

$$\hat{l}_i = \int_\lambda \hat{S}(\lambda)F_i(\lambda)P(\lambda)d\lambda. \quad (6)$$

$P(\lambda)$, $F_i(\lambda)$, and $\hat{S}$ were represented discretely at 5-nm intervals. The estimated percent transmittance

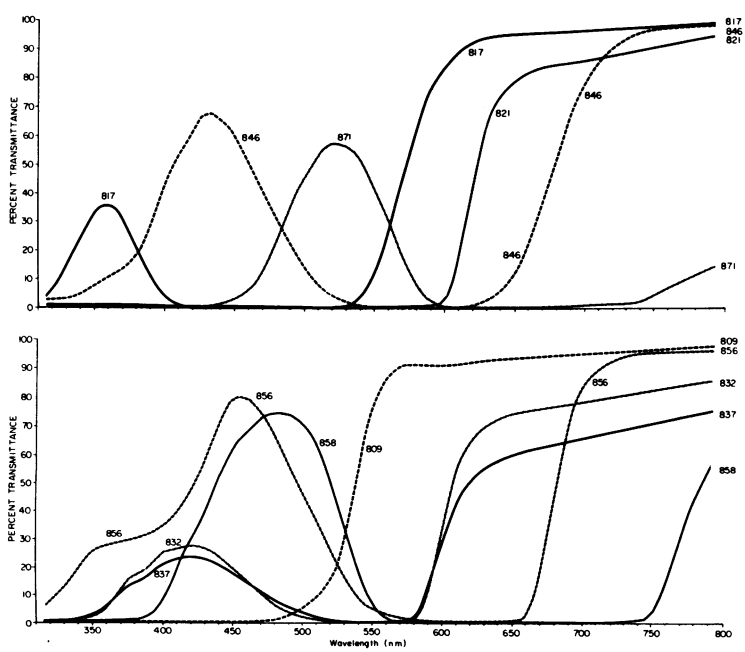

FIG. 1. *Light transmission characteristics of the absorption filters. Numbers on graph correspond to the filter numbers.*

is then:

$$\hat{T}_i = (\hat{I}_i/\hat{I}_0) \times 100. \quad (7)$$

$\hat{S}$ (the estimated spectrum) was arbitrarily chosen to be a Gaussian curve of mean μ and standard deviation σ.

In some simulations, a sum of two Gaussians:

$$S(\lambda) = ag_1(\lambda) + bg_2(\lambda)$$

was employed, where g_1 and g_2 are Gaussians, $0 < a < 1$, and $b = 1 - a$.

An optimum μ and σ for S were selected by the following procedure for each filter. (i) A value of σ was chosen arbitrarily. (ii) The value of μ was then altered to reduce the absolute difference $|T - \hat{T}|$ to a value less than an arbitrary predetermined limit (0.2).

Steps (i) and (ii) were then repeated for different values of σ ranging from 10 to 120 nm.

For filter number i, the best value of μ according to step (ii) was called μ_i. The optimum μ for all the filters for any σ was selected as the median μ_i. The optimum σ was the one that yielded minimal dispersion of the μ_i about the median. The computer simulation was executed on IBM 360 and XDS Sigma 5 computers.

RESULTS

The kinetics of light emission from phagocytizing PMNs in an unfiltered vial (Fig. 2) is similar to other published results (2). Since the kinetics of emission from the nine filtered vials (not shown) were similar to that of the unfiltered vial, and the relative amount of light transmitted though any filter (i.e., the nine T_i values) remained nearly constant with time, the PMN emission spectrum did not change after the initiation of phagocytosis.

Figure 3 shows the rate of CL from the MPO-H_2O_2-Cl^- reaction in an unfiltered vial. Control experiments with either MPO alone or H_2O_2 plus Cl^- did not produce significant amounts of light. Allen (1) found a similar emission curve with this system. Again, the kinetics of light emission for the nine filtered vials (not shown) were similar to that of the unfiltered vial, and each T_i remained nearly constant with time, indicating that the spectrum of the MPO-H_2O_2-Cl^- system also was constant.

To compare the emission spectra of the MPO and PMN systems, the relative amount of light transmitted through the nine filters (T_i) was examined. By using the integrated curve of light emission for the first 6 min of the MPO experiments and 67 min of the PMN experiments, the T_i values were calculated (Fig. 4). The data obtained for the MPO system are strikingly similar to those from the PMNs.

Although the estimated spectrum ($\hat{S}$) (Fig. 5) was constrained to be a Gaussian curve in the simulation, it is clear that the actual spectrum (S) need not be Gaussian in form. However, the results of the simulation indicate that μ and σ can be chosen to yield a Gaussian curve that effectively resembles the actual spectrum in the sense that low values of $|T_i - \hat{T}_i|$ are obtained for almost all the filters. The fact that the $\hat{T}_i$ values (Table 1) for some filters such as 809, 817, and 871 do not give results consistent with those of the other filters indicates that the actual spectrum is not exactly identical to a Gaussian curve. The optimum value of μ was 568 nm for the PMNs and 577 nm for the MPO system, whereas σ was 60 nm for both.

With a somewhat different method, Cheson et al. (5) estimated the CL spectrum of phagocytizing PMNs. We used a discrete (5-nm interval) representation of the reported spectrum in the computer simulation described above. This representation was substituted into equations 5 and 6 as the estimated spectrum, $\hat{S}$. The esti-

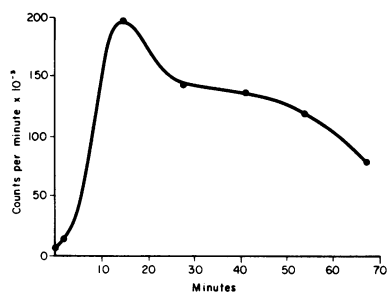

FIG. 2. *Light emission from PMNs after the addition of opsonized zymosan particles.*

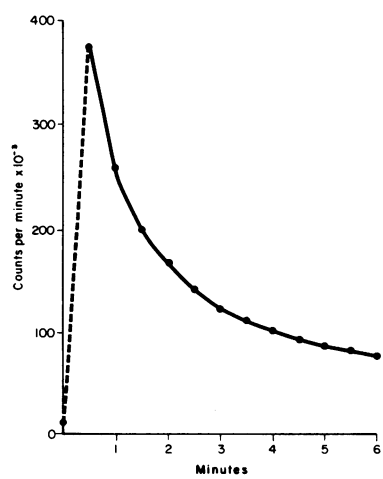

FIG. 3. *Light emission from the MPO-H_2O_2-Cl^- mixture. The dashed line connects the background value of MPO with the first CL value after the addition of H_2O_2 and Cl^-.*

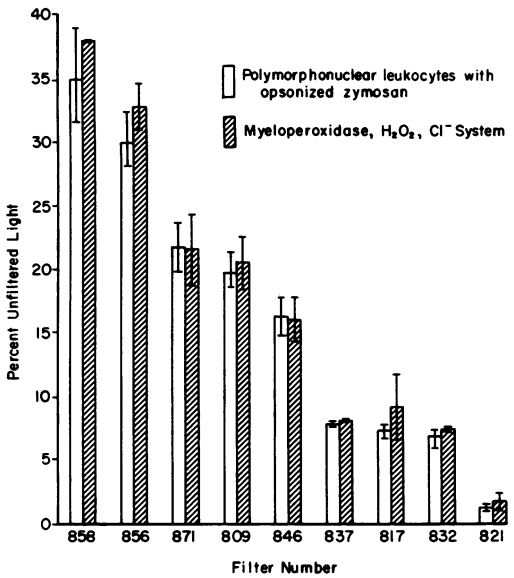

FIG. 4. *Comparison of the light transmission by the PMN and MPO system with nine absorption filters. Percent unfiltered light (T_i) was calculated by the following formula: $T_i = (L_i/L_0) \times 100$. The height of each bar represents the average of three observations for the PMN system and two for the MPO system. The brackets show the range for each measurement.*

mated percent transmittance ($\hat{T}_i$) for each filter used in this experiment, based on the reported spectrum, was then calculated according to equation 7. The values of $\hat{T}_i$ obtained (Table 1) were similar to the results for MPO and PMN CL calculated from our data.

DISCUSSION

The kinetics of CL from phagocytizing PMNs and MPO-H_2O_2-Cl^- were noted in the present study to be similar to other published reports (1, 2). When emission spectra were simulated from the filter data, as described in Materials and Methods, the MPO and PMN curves were virtually identical and, therefore, only the MPO curve is shown (Fig. 5). Similarity of the MPO and PMN curves would be expected from the data shown in Fig. 4. Since the MPO-H_2O_2-Cl^- system is a known $^1O_2$-producing system (Rosen and Klebanoff, Fed. Proc. 35:1391, 1976), the observed similarity of the emission spectra of the two systems strongly suggests that $^1O_2$ is responsible for the CL of PMNs. Rosen and Klebanoff (10), however, have shown that MPO-deficient PMNs retained the capacity to emit light after phagocytosis, although the amount of light produced in the early postphagocytic period was reduced. It is quite possible that $^1O_2$ is produced in MPO-deficient PMNs by other reactions, such as the disproportionation of $\cdot O_2^-$. If the light emission spectrum of MPO-deficient PMNs could be determined, it might be possible to resolve this question.

Although $^1O_2$ is probably responsible for the CL of PMNs, the present study and that of Cheson et al. (5) show that it is not due solely to its relaxation to ground state triplet oxygen and the emission of light. This reaction would release the energy of $^1O_2$ in the form of light, which is not likely to have a bactericidal effect.

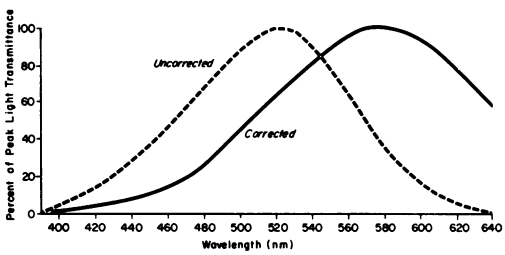

FIG. 5. *Simulated (estimated) light emission curve for the MPO-H_2O_2-Cl^- system. The uncorrected curve represents the effect of not including the PMT quantum efficiency (P) in deriving the estimated spectrum. The best fit for the data when no PMT correction was made was a curve composed of the sum of two Gaussians. The best fit when the correction was included was a single Gaussian of $\sigma = 60$ nm, which for the MPO-H_2O_2-Cl^- system had $\mu = 577$ nm.*

TABLE 1. *T_i and $\hat{T}_i$ from the light emission of phagocytizing PMNs*

Filter no.	Present study[a]		Previously published data[b] — $\hat{T}_i$ (estimated)
	T_i (measured)	$\hat{T}_i$ (estimated)	
858	35.0	40.2	47.5
856	30.0	33.5	43.5
871	21.8	31.1	26.0
809	19.7	32.8	20.6
846	16.3	16.9	26.3
837	7.9	7.3	9.6
817	7.3	12.6	7.8
832	6.9	7.4	9.7
821	1.3	1.4	0.9

[a] Best-fit Gaussian curve for the data from the PMNs in the present study had a $\mu = 568$ nm and $\sigma = 60$ nm.

[b] $\hat{T}_i$ values were calculated from the published spectrum of Cheson et al. (5). This spectrum was used in place of the estimated spectrum ($\hat{S}$) in equations 5 and 6. The $\hat{T}_i$ values were then calculated with equation 7.

Instead, it probably produces light through intermediate reactions that biochemically alter critical bacterial structures or enzymes by oxidation. Since the MPO-H_2O_2-Cl^- system lacks a substrate such as bacteria or zymosan for $^1O_2$ to act upon, the MPO or contaminating proteins may serve as the substrate. This would explain why the emission spectra are different from that of direct $^1O_2$ light (R. Brown and E. Ogryzlo, Proc. Chem. Soc., p. 117, 1964). In an unpublished observation with the same filter system described in this study, $^1O_2$ generated by H_2O_2 and NaOCl produced light with a longer wavelength than that observed in the PMN and MPO systems.

It is possible, however, that direct $^1O_2$ light contributes to the total CL of the PMN if some of the $^1O_2$ does not participate in other reactions. This question cannot be resolved at the present time because the emission spectrum is not precisely defined by either the present or the previously published study (5). Also, neither study used a PMT that was sufficiently sensitive in the range of the two major peaks of the $^1O_2$ emission spectrum, which are at 633 and 704 nm (Browne and Ogryzlo, Proc. Chem. Soc., p. 117, 1964). Nevertheless, it is interesting that, within the effective range of the PMT, both studies show peak light emission near 580 nm, which is one of the emission peaks of $^1O_2$ light.

Our data are in general agreement with the study of Cheson and co-workers (5). The peak of light emission in both studies is approximately the same, and both curves are broad. Although the curve that we have constructed is Gaussian in configuration, it represents only an approximation of the actual spectrum. Further definition of the light source in phagocytizing PMNs and its relationship to the bactericidal process will probably depend on examining isolated cell-free systems with equipment better suited to scanning the entire visible and infrared spectrum.

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