Analysis of the Bimodal Chemiluminescence Pattern Stimulated in Human Neutrophils by Chemotactic Factors

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Chemotactic factors, which are important in attracting neutrophils to inflammatory sites, have also been shown to stimulate oxidative metabolism, resulting in increased chemiluminescence and release of superoxide anion (O_2^-). We observed a unique bimodal chemiluminescence pattern upon stimulation with either the complement-derived factor C5a or formyl-methionyl-leucyl-phenylalanine. A sharp peak of activity occurred within 1 to 2 min, and a second more extended peak was seen between 3 and 6 min. Enhancement of both peaks occurred when the cells were pretreated with cytochalasin B. Expression of both peaks was found to be related to cell density, and expression of the second peak was not dependent upon extracellular metabolites released during the first peak. Cells preincubated in luminol and then thoroughly washed responded with only a single peak coincident with the second peak. Together these findings indicate that the first peak is extracellular in origin, whereas the second peak is cell associated. Studies with scavengers of oxygen intermediates and inhibitors of myeloperoxidase suggest that the first peak requires O_2^- , H_2O_2 , and myeloperoxidase for the oxidation of luminol, which may occur in part through the formation of HOCl as well as through a non-HOCl-mediated mechanism. Evidence for a non-HOCl-mediated mechanism comes from experiments in which luminol, myeloperoxidase, and O_2^- generated by xanthine-xanthine oxidase produce luminescence in the absence of chloride ion. These studies provide further insight into the sequence of events which occur during the stimulation of neutrophils with chemotactic factors and the nature of neutrophil chemiluminescence.

Chemotactic factors have been shown to be important mediators of the inflammatory process. In addition to stimulating the directional locomotion of neutrophils (33), certain chemotactic factors have been shown to directly stimulate superoxide anion (O_2^-) release (15, 24, 39, 46) and chemiluminescence (8-11, 16, 24, 42, 48). Chemotactic factors also enhance the production of oxygen intermediates, such as O_2^- , hydrogen peroxide, singlet oxygen, and hydroxy radical after stimulation with other agents (3, 8, 43). Production of O_2^- via NADPH oxidase, which is putatively the source of these oxygen intermediates (5), is important in mediating the bactericidal action of neutrophils (21). The purpose of this type of stimulation by chemotactic factors is not understood. It has been suggested that release of O_2^- by neutrophils stimulated with chemotactic factors may be important in amplifying the inflammatory response by generating chemotactic lipids (29). The release of oxygen intermediates may also be directly involved in tissue damage (28).

The oxygen intermediates produced by neu-

trophils can be measured indirectly by quantitating the light emission, or chemiluminescence, that occurs when excited electrons present in these intermediates return to ground state and release photons (2). Recently, many investigators have utilized the compound luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) to amplify the chemiluminescence response (1, 8-12, 16, 17, 30, 40, 42, 48). This luminol-enhanced chemiluminescence has been shown to be dependent upon a myeloperoxidase (MPO)-mediated reaction (10-12, 40). In addition, it has recently been suggested that MPO mediates the formation of hypochlorous acid, which can react with luminol to produce light (12). Previous studies from this laboratory (42) and others (10, 11) have shown that neutrophils stimulated with the chemotactic factors C5a and formyl-methionyl-leucyl-phenylalanine (f-MLP) exhibit a unique bimodal pattern of chemiluminescence. In addition, it has recently been shown that interaction of NK cells with their targets also results in a luminol-dependent, bimodal chemiluminescence response (17).

The purpose of the present study was to examine the requirements for the expression of this bimodal pattern of chemiluminescence in an effort to understand the nature of activation of neutrophils by chemotactic factor. In this report we demonstrate that the two peaks of chemiluminescence stimulated by chemotactic factors are composed of a first peak that is an extracellular response and a second peak that is cell associated. These peaks are interrelated and dependent upon MPO. The formation of hypochlorous acid may be involved, but not required, for this type of chemiluminescence response.

MATERIALS AND METHODS

Reagents. Superoxide dismutase (SOD; 3,000 U/mg of protein), sodium azide, L-ascorbic acid, hydroquinone, sodium benzoate, DL-histidine, valine, luminol, colchicine, cytochalasin B (CB), methimazole, 4,4'-diisothiocyano-2,2'-disulfonic acid stilbene (DIDS), *O*-dianisidine, and xanthine oxidase (XO) were obtained from Sigma Chemical Co. (St. Louis, Mo.). 1,4-Diazabicyclo(2.2.2)octane (DABCO) was obtained from Aldrich Chemical Co. (Milwaukee, Wis.). Catalase (75,000 U/mg of protein) and xanthine (X) were obtained from Calbiochem-Behring (La Jolla, Calif.). H_2O_2 was obtained from J. T. Baker Chemical Co., (Phillipsburg, N.J.).

Chemotactic factors. Purified C5a preparations were obtained from fresh normal human serum after its activation with zymosan in the presence of 1 M ϵ -amino-caproic acid (Sigma). C5a was prepared by the method of Fernandez and Hugli (13). f-MLP (Sigma) was stored as a 10 mM stock concentration in dimethyl sulfoxide at -20°C . Just before use, the stock solution was diluted to the appropriate concentration in 0.01 M phosphate-buffered saline (pH 7.4).

Neutrophil preparation. Normal human polymorphonuclear leukocytes (PMNs) were prepared from heparinized peripheral blood by centrifugation through Ficoll-Hypaque as previously described (43). The cell pellet containing erythrocytes and PMNs was suspended in Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.) and mixed with 1 ml of Plasmagel (HTI, Buffalo, N.Y.) per 3 ml of blood. The leukocyte-rich supernatant obtained after 30 min of incubation at 37°C was then centrifuged, and the cell pellet was mixed with 4°C distilled water for 30 s to lyse residual erythrocytes. Cells were then washed twice and suspended in Hanks balanced salt solution. The final preparation contained greater than 90% PMNs.

Chemiluminescence assay. Neutrophil chemiluminescence assays were performed in a Searle Isocap 300 β scintillation counter (Nuclear Chicago, Chicago, Ill.) set out of coincidence, as previously described (42). Chemotactic factors were added to 5×10^6 PMNs in 1 ml of phosphate-buffered saline with 1 mM Ca^{2+} and Mg^{2+} (pH 7.4) in the presence of 100 nM luminol. Samples were counted alternately at ambient temperature with a control at 0.1- or 0.2-min intervals for 10 to 15 min.

Superoxide anion assay. Superoxide anion generation was determined by measuring SOD-inhibitable cytochrome *c* reduction by the change in absorbance

at 550 nm. The method used here was a modification of the technique of Pick and Mizel (34) with microtiter plates in which absorbance was measured with an automated enzyme-linked immunosorbent assay reader (Dynatech Laboratories, Inc., Santa Monica, Calif.). Chemotactic factors were added to 5×10^5 PMNs in 80 μM cytochrome *c* (Sigma) with a final volume of 200 μl per well. The absorbance at 550 nm was then monitored at 10-s intervals for 10 to 12 min. The O_2^- produced was calculated by using a molar extinction coefficient of $21 \times 10^3 \text{ M per cm}$.

MPO release assay. Measurement of MPO release from neutrophils stimulated with chemotactic factors was done as previously described (45). PMNs were pretreated in either Hanks balanced salt solution or Hanks balanced salt solution plus 2.5 μg of CB per ml for 10 min at 37°C . The cells were then centrifuged at $300 \times g$ and suspended in phosphate-buffered saline with 1 mM Ca^{2+} and 1 mM Mg^{2+} . Triplicate wells of a microtiter plate (Falcon Plastics, Oxnard, Calif.) containing 5×10^5 PMNs in 100 μl were then stimulated at 25°C for various times with $5 \times 10^{-7} \text{ M f-MLP}$. This was then followed by the addition of 25 μl of a 0.2 M phosphate buffer (pH 6.2) and 25 μl of an equal mixture of 3.9 mM *O*-dianisidine HCl and 15 mM H_2O_2 . After a 10-min incubation at 25°C , 25 μl of 1% NaN_3 was added to stop the reaction, and the plate was read on an automated enzyme-linked immunosorbent assay reader measuring optical density at 450 nm.

Production of O_2^- and chemiluminescence by X-XO. The quantity of O_2^- produced by X-XO was determined by measuring cytochrome *c* reduction at 550 nm as described above. XO (0.76 U/mg of protein) was dialyzed for 15 h against 0.1 M phosphate buffer (pH 7.4). The protein concentration was then determined by the method of Lowry (25) to be 9.3 mg/ml. O_2^- production by this system was determined by the addition of various amounts XO to a well containing 80 μM cytochrome *c* and 1 mM X. The absorbance at 550 nm was then recorded at 10-s intervals. The O_2^- produced by each of the concentrations of XO was determined from the slope of the line generated. Comparison of the nanomoles of O_2^- produced per minute by each concentration of XO yielded a straight line from which it was determined that each 100 mU of XO produces about 2.4 nmol of O_2^- per min.

Luminol-dependent chemiluminescence of the X-XO system was performed in 0.1 M phosphate buffer (pH 7.4) by the addition of 71 mU of XO to a vial containing 10^{-7} M luminol and 1 mM X. The sample was then counted repetitively for 0.1-min intervals. The neutrophil particulate fraction that was added to this assay was prepared as previously described (7).

RESULTS

Chemiluminescence response of neutrophils stimulated with chemotactic factors. The addition of either f-MLP or purified C5a to 5×10^6 neutrophils in the presence of luminol (100 nM) results in a rapid bimodal stimulation of chemiluminescence. The average first peak response with 10^{-7} M f-MLP occurred at $0.84 \pm 0.2 \text{ min}$ ($x \pm 1$ standard deviation) in 15 experiments with different cell preparations. This initial response subsides by 2 min and is followed by the appear-

ance of a second, broader peak of activity occurring between 3 and 7 minutes ($\bar{x} \pm 1$ standard deviation, 5.0 ± 0.7 min; $N = 15$). A similar time frame was observed with C5a. Representative dose response curves with f-MLP and purified C5a are shown in Fig. 1. Although the magnitude of the chemiluminescence response varied between cell preparations, the time at which the peaks occurred was consistent. Cells assayed in phosphate-buffered saline without Ca^{2+} or Mg^{2+} (data not shown) gave a lower response, but still showed a bimodal pattern. Pretreatment of the cells with $2.5 \mu\text{g}$ of CB substantially enhanced the expression of the first peak by $499 \pm 199\%$ and the second peak by $366 \pm 126\%$ ($\bar{x} \pm 1$ standard deviation, $n = 7$).

Effect of cell density and presence of factor upon the chemiluminescence pattern stimulated by f-MLP. In the experiments testing the effect of cell density and the presence of factor upon chemiluminescence, the total number of cells (5×10^6) in each vial was kept constant, and the volume in which they were stimulated was varied, so that cell concentrations ranged from 5×10^6 cells per ml to 1.25×10^6 cells per ml. In each case the concentration of f-MLP and luminol remained the same. The expression of the two peaks was dependent upon cell concentration (Fig. 2). As the cell density decreased, the magnitude of the first peak decreased, and the second peak increased proportionately. Al-

though the relative amplitude of each peak was affected by cell density, there was no change in the temporal expression of this pattern.

To determine whether the continued presence of f-MLP was required for the expression of the second peak, cells were stimulated with f-MLP in buffer only. The control preparation was stimulated simultaneously with f-MLP in luminol and counted continuously at 0.2-min intervals. At 1 min, the cells stimulated in buffer alone were centrifuged for 30 s at $1,000 \times g$, and the supernatant was removed. These cells were then added to a vial containing buffer and luminol without f-MLP. This sample was then counted alternately with the control sample containing neutrophils stimulated with f-MLP in the presence of luminol (Fig. 3). The preparations in which the factor had been removed exhibited a peak that was coincident with the second peak obtained with the sample where f-MLP was present throughout, although the response was always slightly lower than the control response (possibly due to cell loss). Other experiments (data not shown) in which the f-MLP-stimulated cells were added to buffer with luminol at a later time (5 to 6 min) showed that the second peak response was already at its apex and the activity declined thereafter coincident with that of the control sample. Although the f-MLP was probably not completely removed in these experiments, the concentration was lowered approxi-

FIG. 1. Bimodal chemiluminescence of 5×10^6 human neutrophils in response to various concentrations of the chemotactic factors C5a and f-MLP in the presence of 100 nM luminol. The data presented in the two panels are from different cell preparations.

FIG. 2. Effect of varying the cell density on the expression of the chemiluminescence response of neutrophils to 10^{-7} M f-MLP. Shown is a representative experiment of four experiments with different cell preparations.

mately 100-fold from 10^{-7} M to 10^{-9} M, a concentration that does not stimulate appreciable activity (Fig. 1). These findings suggest that the continued presence of the initial concentration of soluble f-MLP or the initial presence of luminol is not required for expression of the second peak.

Effect of exposure of neutrophils to luminol before stimulation with f-MLP. Since it has been shown that luminol will penetrate the cell (4), further experiments were performed in which PMNs were incubated in 10^{-6} M luminol for 15 min at 37°C and washed three times before stimulation with 10^{-7} M f-MLP. This cell preparation was assayed for chemiluminescence simultaneously with a control preparation incubated in medium alone and a luminol-pretreated preparation stimulated with f-MLP in the presence of 10^{-7} M luminol. As shown in Fig. 4B, neutrophils preincubated with luminol (10^{-6} M) and washed three times responded to f-MLP stimulation with only a single peak response corresponding to the second peak of chemiluminescence observed in the control cells stimulated in the presence of extracellular luminol (10^{-7} M) (Fig. 4A). Furthermore, neutrophils preincubated in luminol and washed three times still produced a bimodal chemiluminescence response when stimulated in the presence of extracellular luminol (Fig. 4C). These experiments indicate that the first peak of chemiluminescence represents an extracellular phenomenon, whereas the second peak is cell associated.

Effect of oxygen radical scavengers and other inhibitors on the expression of the bimodal chemiluminescence pattern. Dismutation of O_2^- (27)

by the addition of SOD (Table 1) resulted in almost complete inhibition of the first peak without significant effect upon the expression of the second peak in cells with or without CB pretreatment. Heat-denatured SOD treated at 100°C for 10 min was without effect. The presence of ascorbic acid, which has been reported to interact with O_2^- (32, 23), also preferentially

FIG. 3. Effect of removal of the factor on the expression of the second peak of chemiluminescence stimulated by f-MLP. PMNs were stimulated with 10^{-7} M f-MLP in buffer only (○), or simultaneously a control preparation was stimulated in 100 nM luminol (●) and counted. At 1 min the buffer only sample was centrifuged ($\downarrow$), the supernatant was removed, and the cell pellet was added to a vial containing buffer and luminol without f-MLP ($\downarrow$) and counted alternately with the control. Shown is a representative example of four experiments done on different cell preparations.

FIG. 4. Evidence for an extracellular first peak and intracellular second peak of chemiluminescence in response to f-MLP. A, Results of cells responding to 10^{-7} M f-MLP in the presence of 10^{-7} M luminol. B, Response of cells that were preincubated for 15 min in 10^{-6} M luminol, washed free of extracellular luminol, and stimulated with f-MLP without additional luminol. C, Response of cells preincubated in 10^{-6} M luminol, washed free of extracellular luminol, and stimulated with f-MLP in the presence of 10^{-7} M luminol. The results shown were obtained with the same cell preparation and are representative of experiments with three different cell preparations.

inhibited the first peak of chemiluminescence without affecting the second. The direct reduction of cytochrome *c* by ascorbic acid precluded its study with the O_2^- assay (22). These results suggest that O_2^- plays an important role in the expression of the first peak.

The addition of catalase to the assay was without significant inhibitory effect in non-CB-treated cells. When CB-treated cells were tested in the presence of catalase, substantial inhibition of chemiluminescence was observed, with the

first peak being inhibited to a greater extent than the second. Catalase that was heat denatured at 100°C for 10 min had no inhibitory effect.

Since hypochlorous acid (HOCl) has been recently implicated in the production of luminol-dependent chemiluminescence (12), taurine, known to react with HOCl (47) and inhibit the MPO- H_2O_2 - Cl^- antimicrobial system (41) was also tested. As shown in Table 1, taurine substantially inhibited the first peak and augmented the expression of the second peak.

Additional scavengers of singlet oxygen and hydroxy radical were tested. As shown in Table 1, hydroquinone, which has been reported to scavenge singlet oxygen (14), also gave an inhibition pattern similar to SOD and ascorbic acid. This effect was much less apparent with CB. To determine whether hydroquinone may be acting to scavenge O_2^- , it was tested in the O_2^- assay and found to be without effect on f-MLP-stimulated O_2^- release with or without pretreatment with CB. The other scavengers of singlet oxygen, DABCO (14, 35) and histidine (26), were without inhibitory effect on either peak. Likewise, the hydroxy radical scavengers tested, benzoate and mannitol (31), were also without inhibitory effect.

As MPO has been implicated as an important component in luminol-dependent chemiluminescence, several MPO inhibitors were tested to determine their effect on the bimodal chemiluminescence pattern stimulated by chemotactic factors. As shown in Table 1, both azide (37) and methimazole substantially inhibited the expression of both peaks of chemiluminescence. This inhibition was more pronounced with the second peak and occurred with and without pretreatment with CB. In contrast, cyanide stimulated a twofold enhancement of the first peak and caused a moderate reduction in the second peak in both CB-treated and untreated cells.

To examine the role of MPO release in the expression of chemiluminescence, neutrophils were pretreated with DIDS (0.1 mM), an anion channel blocker that at this concentration inhibits degranulation without affecting O_2^- production (22). Inhibition of MPO release by f-MLP-stimulated neutrophils was confirmed in the exocytosis assay. Likewise, DIDS was tested for its effect upon f-MLP-stimulated O_2^- release and was found to be not inhibitory (Table 1). As shown in Table 1, DIDS substantially inhibited expression of the f-MLP-stimulated bimodal chemiluminescence response only in CB-treated cells where exocytosis occurs.

Kinetics of O_2^- and MPO release from f-MLP-stimulated neutrophils. Since O_2^- and MPO appear to be important components in the luminol-dependent chemiluminescence (11, 12, 36, 40), the kinetics of their release were examined for

TABLE 1. Effect of inhibitors on the production of chemiluminescence or superoxide anion from neutrophils stimulated with f-MLP

Inhibitor ^a	Chemiluminescence ^b				O ₂ ^{-b}	
	Without CB		With CB ^c		Without CB	With CB ^c
	Peak 1	Peak 2	Peak 1	Peak 2		
SOD (100 µg/ml)	12 ± 4	98 ± 20 (4)	27 ± 8	92 ± 76 (4)		
Ascorbate (10 mM)	9 ± 2	130 ± 28 (4)	11 ± 6	113 ± 14 (3)		
Catalase (200 µg/ml)	81 ± 11	125 ± 35 (4)	27 ± 12	56 ± 4 (3)	104 ± 2	103 ± 6 (3)
Taurine (10 mM)	58 ± 1	193 ± 51 (3)	39 ± 7	192 ± 14 (4)	104 ± 12	89 ± 8 (3)
DABCO (1 mM)	185 ± 15	125 ± 8 (4)	255 ± 17	106 ± 12 (4)		
Hydroquinone (1 µM)	13 ± 5	127 ± 18 (4)	68 ± 11	91 ± 7 (4)	104 ± 10	99 ± 10 (3)
Histidine (10 mM)	129 ± 18	144 ± 11 (4)	230 ± 70	135 ± 5 (4)		
Azide (1 mM)	55.8 ± 6	17.0 ± 5 (4)	15 ± 3	9 ± 3 (3)	83 ± 4	73 ± 4 (3)
Methimazole (2 mM)	26.3 ± 6	18.6 ± 5 (3)	24 ± 7	18 ± 4 (3)	90 ± 4	81 ± 5 (3)
Cyanide (1 mM)	219 ± 18	19 ± 8 (4)	198 ± 31	47 ± 10 (3)	124 ± 9	96 ± 6 (3)
DIDS (0.1 mM)	136 ± 30	137 ± 23 (4)	31 ± 8	42 ± 12 (4)	116 ± 4	117 ± 15 (3)

^a PMNs were not preincubated in the inhibitor, but rather the inhibitor was only present at the time of stimulation. When DIDS was used, the cells were preincubated for 20 minutes at 37°C prior to stimulation.

^b Results are expressed as the mean percent of control ± 1 standard error of the mean. The numbers within parentheses indicate the numbers of experiments performed with different cell preparations.

^c PMNs assayed after a preincubation in 2.5 µg of CB per ml for 10 min at 37°C.

comparison with the appearance of chemiluminescence in f-MLP-stimulated neutrophils. As determined by the O-dianisidine assay for MPO release (45), no release of MPO was detected in the absence of CB pretreatment. When the cells were pretreated with CB, 32 ± 8% of the total MPO was released; 95% of this release occurred within 1 min, and 100% occurred within the first 2 min of stimulation. When O₂⁻ release was examined kinetically by measuring cytochrome c reduction, detectable O₂⁻ was released from non-CB-treated neutrophils; 75% of this occurred within the first 2 min of stimulation. CB pretreatment resulted in enhanced detection of O₂⁻ with the majority of the production occurring within the first 2 min. These findings are in agreement with other studies (18, 24) and indicate that the first peak of chemiluminescence coincides with the external release of O₂⁻ and MPO in CB-treated neutrophils and the release of O₂⁻, but not MPO, in non-CB-treated cells.

Luminol-dependent chemiluminescence of an O₂⁻-generating system. As luminol-dependent chemiluminescence appears to require both oxygen radicals and MPO, we further examined this response with X-XO as a source of O₂⁻. These experiments were performed at the physiological pH of 7.4 in the absence of exogenous chloride ion required for the formation of hypochlorous acid. When 71 mU of XO, known to produce 1.7 nmol of O₂⁻ per min, was added to a vial containing 1 ml of 0.1 M phosphate buffer (pH 7.4), 1 mM X, and 10⁻⁶ M luminol, no chemiluminescence response was observed. However, when 25 µg of neutrophil particulate

fraction protein was added to this assay as a source of MPO, a burst of chemiluminescence was observed (>7 × 10⁵ counts/0.1 min), which decayed rapidly to 2 × 10⁴ counts/0.1 min within 3 to 4 min. When either SOD (50 µg/ml) or catalase (50 µg/ml) was added to this assay, complete inhibition of the response occurred. These findings indicate that luminol can interact with MPO and oxygen radicals in the absence of chloride to produce chemiluminescence and that this response is dependent upon the presence of both O₂⁻ and H₂O₂.

DISCUSSION

Hatch et al. (16) reported that formyl-methionyl peptides stimulate chemiluminescence; this has since been confirmed by other investigators (3, 8–11, 24, 42, 48). As indicated by studies from this laboratory (42) and others (10, 11), the chemiluminescence response stimulated by the chemotactic factors f-MLP and C5a is bimodal. The current study demonstrates that the expression of a bimodal pattern of chemiluminescence is directly dependent upon cell density. A minimum cell density is required to produce the first peak (Fig. 2). The amplitude of this peak increases proportionally as the cell concentration is increased beyond this threshold. On the contrary, as the cell density is increased the second peak decreases simultaneous with an increase in the first peak. A similar effect of increasing cell densities has been reported in the measurement of chemotaxis (44) and suggests that cell interactions may be important in the expression of these functions. During the course of this study,

Dahlgren and Stendahl (10) showed that the expression of both peaks of chemiluminescence stimulated by f-MLP could be modulated in the same way as has been presented here at higher cell densities by *in vitro* preincubation of the cells at 22°C. In their study, an increase in the first peak of chemiluminescence was related to an increased spontaneous release of MPO. This interpretation is consistent with enhancement of the extracellular first peak observed here with higher cell densities, since extracellular MPO concentrations under these conditions would be greater. In addition, since MPO has been implicated in terminating the respiratory burst (19), concentrations of metabolites, such as HOCl, would be greater at higher cell densities and may result in the autooxidation of the cells causing inhibition of the second phase of chemiluminescence. That HOCl plays a role in the suppression of the second peak is suggested by the augmentation of the second peak observed in the presence of taurine.

Other experiments in which cells were washed free of metabolites and soluble factor present during the first peak response indicate that expression of the second peak was independent of the presence of luminol and soluble products released during the first peak. This suggests that the second peak may be a secondary cellular response triggered by factor bound to the cell surface or internalized. This is consistent with the studies shown in Fig. 4 indicating that the first peak is an extracellular event, whereas the second peak is an intracellular event.

Pretreatment with CB augmented both peaks of chemiluminescence, with a greater effect on the first peak. This may be due to enhanced extracellular release of both MPO (6, 18) and O_2^- (24). Alternatively, CB could be enhancing chemiluminescence by increasing the proportion of cells responding to f-MLP (38), as shown to occur in assays of membrane depolarization.

The experiments which show that cells preincubated in luminol and then thoroughly washed respond with a single peak coincident with the second peak (Fig. 4) indicate that the first peak is indeed an extracellular event and suggest that the second peak is an internal or cell-associated phenomenon. A similar type of response has been reported (4) in which higher concentrations of luminol alone ($>10^{-5}$ M) were shown to stimulate cell-associated chemiluminescence response that occurs between 5 and 10 min. Although the second peak appears to be a cell-associated or internal event, it may not be related to internalization of receptor-ligand complexes known to occur during this time period (20), since the second peak occurred with CB treatment, which interferes with internalization. A recent report by Dahlgren and Stendahl (11)

supports an extracellular origin of the first peak by demonstrating that MPO-deficient PMNs that do not normally exhibit luminol-dependent chemiluminescence will indeed respond to f-MLP stimulation when exogenous MPO is added to the system. The response is a single peak coincident with the first peak and consistent with an extracellular event.

The use of various scavengers of oxygen intermediates indicates that the first peak of chemiluminescence is dependent upon the interaction of both O_2^- and H_2O_2 with luminol and MPO. Inhibition with SOD and ascorbate suggests the involvement of O_2^- . The inhibition with catalase was much more pronounced with CB-treated cells and may reflect the better access of catalase to externally released H_2O_2 or, alternatively, a greater role for H_2O_2 in the chemiluminescence of CB-treated cells. The lack of inhibition by mannitol or benzoate suggests that hydroxy radical is not involved in this response. Likewise, no definite role for singlet oxygen can be proposed, since inhibition was observed with hydroquinone, but not with DABCO or histidine. Inhibition by this concentration of hydroquinone (1 μ M) is contradictory to its role as a singlet oxygen scavenger, since it has been reported to be a less potent 1O_2 scavenger than DABCO (14), which did not inhibit even at a 1 mM concentration.

The importance of MPO in luminol-dependent chemiluminescence has been established by several investigators (11, 12, 40). Studies with azide and methimazole support a role for MPO in both peaks. The enhancement seen with cyanide is an enigma and can only in part be explained by the ability of cyanide to augment O_2^- release in non-CB-treated neutrophils (Table 1) (39). Hypochlorous acid produced as a result of the MPO- H_2O_2 -Cl interaction may be involved in the luminol-dependent chemiluminescence (12), since taurine, which is known to form a stable chloramine upon reaction with HOCl (47), partially inhibited the first peak and augmented the second peak. These data suggest a role for HOCl in the first peak and imply that HOCl may suppress the second peak. A similar enhancement of the second peak was also observed at low cell densities (Fig. 2), where extracellular HOCl concentrations would also be decreased. Since taurine only partially inhibited the first peak, HOCl cannot account for all of the chemiluminescence produced. Indeed, other mechanisms of chemiluminescence are supported by the observation that the NADPH oxidase stimulates luminol-dependent chemiluminescence in the absence of chloride ion (7). The experiments presented here with a chloride-free X-XO-luminol-MPO system to produce chemiluminescence support this possibility. This system re-

quires the presence of both H_2O_2 and O_2^- , as does the first peak of chemotactic factor-stimulated chemiluminescence. This is consistent with a mechanism proposed by Misra and Squatrito (30) of the peroxidase-catalyzed chemiluminescence of luminol, which does not involve HOCl, but requires both O_2^- and H_2O_2 for the formation and oxidation of a luminol radical.

The role of O_2^- and MPO in the first peak of chemiluminescence coincides well with extracellular production of O_2^- (46) and the release of MPO from CB-treated cells (18). Interestingly, in agreement with other studies (18), no MPO release was detected in non-CB-treated cells, although bimodal chemiluminescence was observed. One explanation is that the *o*-dianisidine assay is not sensitive enough to measure the small amounts of MPO released. Support for this comes from a recent study by Dahlgren and Stendahl (10) in which supernatants from PMN suspensions were shown to contain MPO as measured with guaiacol.

In summary, this study of the bimodal chemiluminescence pattern stimulated by chemotactic factors demonstrate that the first peak is of extracellular origin and requires O_2^- , H_2O_2 , and MPO for the oxidation of luminol. This may occur in part through the formation of HOCl, as well as through a non-HOCl-mediated mechanism. The second peak represents a cell-associated response possibly related to the internalization of the receptor-ligand complex after stimulation.

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