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Role of Myeloperoxidase in Luminol-Dependent Chemiluminescence of Polymorphonuclear Leukocytes

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When polymorphonuclear leukocytes (PMNL) and soluble or particulate matter interact, the cells produce chemiluminescence, linked to activation of the oxidative metabolism of the cells. PMNL isolated from a patient with a myeloperoxidase deficiency were found to produce almost no luminol-dependent chemiluminescence, despite a pronounced production of superoxide anions (O_2^-). The chemotactic peptide formylmethionyl-leucyl-phenylalanine induced a two-peak chemiluminescence response in control PMNL. The response was modified, both in magnitude and in time-course, when the cells were incubated at 22°C for 120 min. Addition of purified myeloperoxidase to the PMNL lacking this enzyme, before stimulus addition, resulted in a chemiluminescence response. In the response to formylmethionyl-leucyl-phenylalanine, only one peak, corresponding to the initial peak of control PMNL, was found. This indicated that luminol-dependent chemiluminescence is dependent on and directly related to the presence of myeloperoxidase in PMNL and that both intra- and extracellularly located myeloperoxidase has to be taken into account when interpreting the cellular response assayed as chemiluminescence.

The generation of chemically reactive molecules, e.g., superoxide anions, hydrogen peroxide, singlet oxygen, and hydroxyl radicals, as a result of respiratory burst activation in polymorphonuclear leukocytes (PMNL) is an essential step in the host defense against microorganisms. Light emission or chemiluminescence of PMNL, a phenomenon related to the respiratory burst activation, was originally described by Allan et al. (1). It has since then been shown that the chemiluminescence response of PMNL is influenced by a number of factors (3, 11, 19). Two mechanisms have been proposed to be responsible for light emission of PMNL, one involving myeloperoxidase (MPO) and the other involving superoxide anion (O_2^-) (20, 27), but the relative importance of O_2^- and MPO may differ, depending on the assay system used (13, 27, 29). The chemiluminescence response of PMNL has usually been traced as one peak of emittance (maximal response after 10 to 20 min) (3, 8, 27, 31), but an initial peak appearing 1 to 3 min after stimulus addition has also been described (6, 12, 18, 23). We have previously shown that the chemotactic peptide formylmethionyl-leucyl-phenylalanine (fMLP) induces a two-peak luminol-dependent chemiluminescence response in PMNL, and proposed that the first peak was due to extracellularly released MPO reacting with hydrogen peroxide (H_2O_2) and that the second peak of light emission could be related directly to cellular production of O_2^- (12).

In this study, we investigated the PMNL response in a patient with an MPO deficiency and found that despite a pronounced O_2^- release from these cells, almost no luminol-dependent chemiluminescence was detected but that a chemiluminescence response could be obtained from these cells after addition of purified MPO.

MATERIALS AND METHODS

PMNL. To obtain PMNL, EDTA-blood from normal healthy laboratory personnel and from a previously described MPO-deficient patient (28) was separated by the method of Bøyum (7). The remaining erythrocytes were removed by hypotonic lysis, and the PMNL were washed twice in Krebs-Ringer phosphate buffer supplemented with 10 mM glucose (KRG; pH 7.2) and suspended (2×10^7/ml) in the same buffer.

Reagents. fMLP, phorbol myristate acetate (PMA), cytochrome *c*, superoxide dismutase (SOD), cytochalasin B (cyt B), and luminol were obtained from the Sigma Chemical Co., St. Louis, Mo. Purified MPO from human leukocytes was a generous gift from Inge Olsson (Lund, Sweden).

Chemiluminescence measurements. Chemiluminescence was measured as described elsewhere (12), with the modification that the number of responding PMNL was reduced. Briefly, measurements were made in a liquid scintillation counter (Isocap 300; Searle-Nuclear, Chicago, Ill.) kept at 22°C, with the coincidence circuit inserted and in the tritium mode (12, 14). Samples for chemiluminescence were obtained by adding 0.6 ml of KRG containing luminol (10^{-8} to 10^{-7}

TABLE 1. MPO-mediated iodination and superoxide production in normal and MPO-deficient PMNL

PMNL	Iodination[a]	Superoxide production			
		PMA		fMLP	
		Rate[b]	Total[c]	Rate[b]	Total[c]
Control	0.40	25.5	77.4	19.3	45.2
MPO-deficient	0.02	23.7	76.6	12.6	54.8

[a] Iodination, in nanomoles of I per 10^6 PMNL per 30 min, assayed during phagocytosis of yeast particles. Mean of two experiments.

[b] Maximum rate expressed in nanomoles of O_2^- per 10^6 PMNL per minute, using PMA (2×10^{-6}) or fMLP (10^{-7} M) as the stimulus. Mean of two experiments.

[c] Total superoxide production after 15 min, expressed as nanomoles of O_2^- per 10^6 PMNL.

M) and 10 μl of PMNL suspension to disposable 4-ml polypropylene tubes. The tubes were put in glass vials, placed in the scintillation counter, and allowed to stand until a stable background of chemiluminescence was obtained (less than 2 min). The light emission from the reactive vials was recorded repeatedly for 6 s. To activate the systems, we added 0.1 ml of a stimulus diluted to appropriate concentration in KRG, stirred the tubes, and recorded the light emission.

Measurement of PMNL iodination. Iodination was carried out essentially as described previously by Olsson et al. (26). The reaction mixture contained 10^6 PMNL, 10% pooled human serum, 30 nmol of sodium iodide (0.5 μCi of ^{125}I), 5×10^7 yeast particles, and KRG to a final volume of 0.5 ml. The tubes were incubated at 37°C, and the reaction was terminated after 30 min with 0.1 ml of 0.1 M sodium thiosulfate solution. We then added 5 ml of cold 10% trichloroacetic acid. After centrifugation, the precipitates were washed three times with 5 ml of trichloroacetic acid. The iodination was expressed as nanomoles of ^{125}I precipitated per 10^6 PMNL per 30 min.

Measurement of O_2^- production. A continuous assay for estimating O_2^- production was utilized essentially as described previously by Cohen and Chovaniec (10), using a double beam spectrophotometer (DB-G; Beckman Instruments, Inc., Fullerton, Calif.) with a temperature regulator (37°C). In a standard assay, both sample and reference cuvettes contained 2.5×10^6 PMNL and 300 nmol of cytochrome *c* in 2 ml of KRG. The reference cuvette furthermore contained 200 U of SOD per ml. At zero time, 0.1 ml of PMA (5×10^{-5} M) or 0.25 ml of fMLP (5×10^{-6} M) was added to both cuvettes, and the absorbance change accompanying cytochrome *c* reduction was monitored at 550 nm. The rate of O_2^- production was calculated from the linear rate of absorbance change, using a millimolar extinction coefficient for cytochrome *c* of 15.5 at 550 nm (24), and was expressed as nanomoles of O_2^- per 10^6 PMNL.

RESULTS

MPO-mediated iodination and O_2^- production. In the initial experiments, we confirmed our previous findings (28) that PMNL from a patient with generalized pustular psoriasis show practically no (<5%) iodination of ingested yeast particles (Table 1). The defective iodination of ingested yeast particles was due to lack of MPO, as earlier shown by immunochemical analysis (28). Upon interaction between MPO-deficient PMNL and PMA or fMLP, no significant reduction of cellularly produced superoxide anions was observed (Table 1). Although the rate of production was slightly impaired, the lag-time or total production did not differ. In fact, the fMLP-activated, MPO-deficient cells continued to produce O_2^- for a longer period of time than normal PMNL did.

Chemiluminescence. When control PMNL preincubated at 22°C for 30 min were exposed to fMLP, the cells responded and produced chemiluminescence. When the time-course of chemiluminescence emission was studied, an initial peak was found within 2 min and a second peak was found around 10 min after stimulus addition (Fig. 1). When MPO-deficient PMNL, prepared

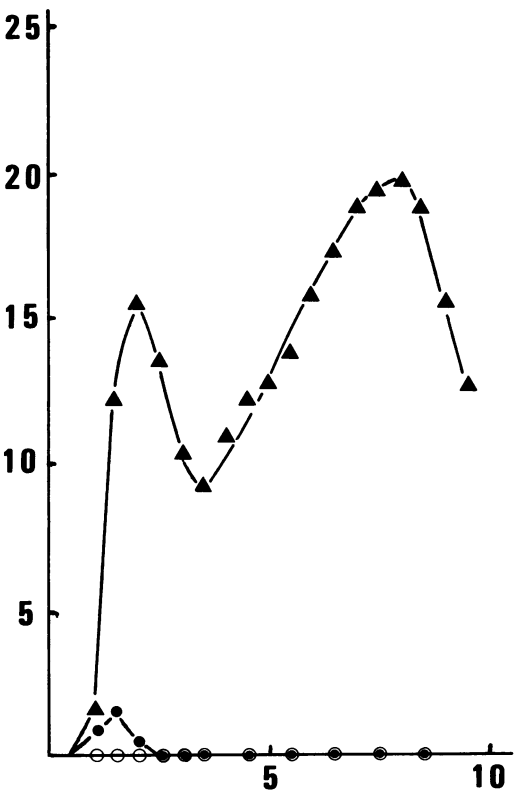

FIG. 1. Time trace of chemiluminescence emitted from PMNL (2×10^5 PMNL; 10^{-7} M luminol) incubated at 22°C for 30 min, when exposed to 10^{-7} M fMLP. Symbols: ▲, control PMNL; ○, MPO-deficient PMNL; ●, MPO-deficient PMNL with 2.5 μg of MPO per ml. Abscissa: Time of study (minutes). Ordinate: Chemiluminescence in counts per minute ($\times 10^2$).

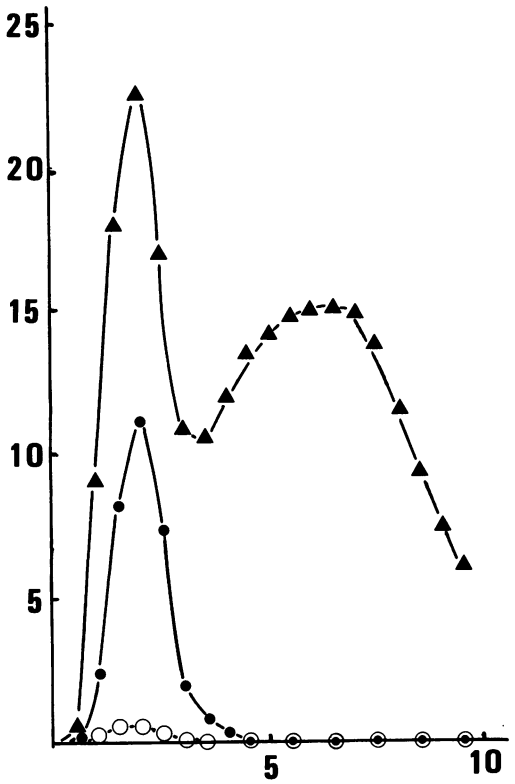

FIG. 2. Time trace of chemiluminescence emitted from PMNL (2×10^5 PMNL; 10^{-7} M luminol) incubated at 22°C for 120 min, when exposed to 10^{-7} M fMLP. Symbols: ▲, control PMNL, ○, MPO-deficient PMNL; ●, MPO-deficient PMNL with 2.5 μg of MPO per ml. Abscissa: Time of study (minutes). Ordinate: Chemiluminescence in counts per minute ($\times 10^3$).

and stored as control cells, were exposed to fMLP, no chemiluminescence response was observed (Fig. 1). Upon preincubation of the cells (120 min at 22°C), changes in the cellular chemiluminescence response to fMLP were observed, in that for control PMNL both peaks increased and for MPO-deficient PMNL a small peak appeared 2 min after stimulus addition (Fig. 2). The interaction between control PMNL and PMA or a particulate stimulus (opsonized yeast particles) gave rise to a pronounced chemiluminescence response from the cells, with a lag phase of 2 to 5 min before any measurable signals were recorded. The peak activity was reached after 15 to 25 min. When MPO-deficient PMNL were exposed to PMA or opsonized yeast particles, no chemiluminescence response was observed (Fig. 3).

Effect of MPO on cellular response. Addition of purified MPO to MPO-deficient PMNL before stimulus addition resulted in a chemiluminescence response when the cells were exposed to fMLP or PMA (Fig. 1, 2, and 3). The cellular response to fMLP was more pronounced for cells stored for 120 min at 22°C than for cells stored for 30 min at 22°C.

Effect of cyt B on cellular response. In the presence of cyt B, the time-course of the chemiluminescence response of control PMNL was modified, in that the relation between the first and the second peak was changed (Fig. 3).

DISCUSSION

The production of highly reactive molecules in PMNL, e.g., superoxide anions, hydrogen peroxide, singlet oxygen, and hydroxyl radicals, as a result of respiratory burst activation is an essential step in host defense against microorganisms, but may also function as a basis for modulation of the phagocyte function (4, 5). The production of these highly reactive molecules can be measured as light emission (chemiluminescence) from PMNL. Chemiluminescence from PMNL has been shown to be influenced by a number of factors (3, 19), and the data on the effect of chemoattractants on the PMNL chemiluminescence response are contradictory (2, 12, 18, 23), both concerning the ability of these substances to induce a response and in the time-course of the response. The chemiluminescence response by PMNL has usually been traced as

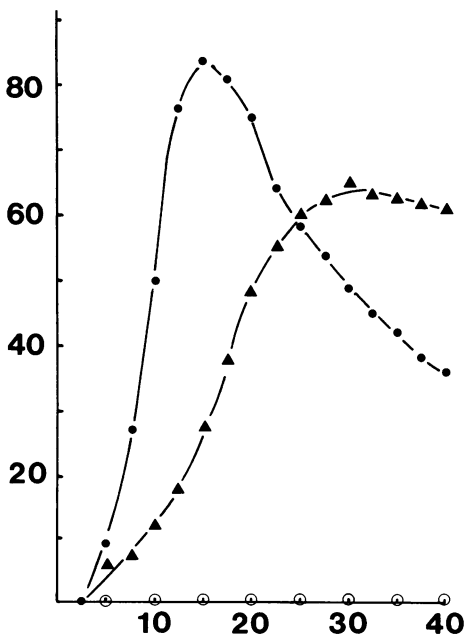

FIG. 3. Time trace of chemiluminescence emitted from PMNL (2×10^5 PMNL; 10^{-8} M luminol) when exposed to 10^{-7} M PMA. Symbols: ▲, control PMNL; ○, MPO-deficient PMNL; ●, MPO-deficient PMNL with 0.25 μg of MPO per ml. Abscissa: Time of study (minutes). Ordinate: Chemiluminescence in counts per minute ($\times 10^3$).

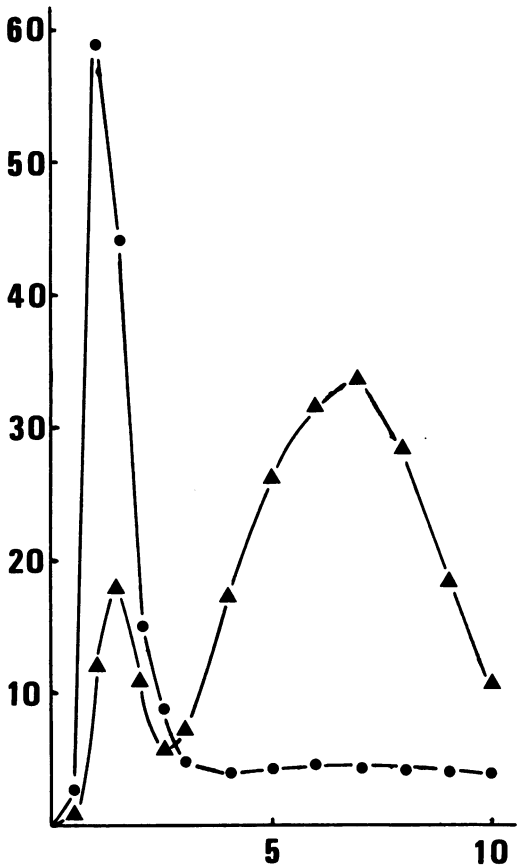

FIG. 4. Time trace of chemiluminescence emitted from PMNL when exposed to 10^{-7} M fMLP in the absence (▲) or in the presence (●) of cyt B (10 μg/ml). Abscissa: Time of study (minutes). Ordinate: Chemiluminescence in counts per minute $\times 10^2$ (▲) or $\times 10^3$ (●).

one peak of emittance with a maximal response obtained 10 to 20 min after stimulus addition (3, 8, 27, 31), but an initial peak appearing within 2 min after stimulus addition has also been described (6, 12, 18, 23). Two mechanisms have been proposed to be responsible for the light emission in PMNL, one involving MPO and the other involving superoxide anion (O_2^-) (20, 27), and we have proposed that the initial peak observed in the PMNL chemiluminescence response is linked to the amount of cellularly released MPO producing chemiluminescence by reacting with H_2O_2 released by the cells as a response to a stimulus (12) and that the second peak observed could be related directly (independent of MPO) to cellular production of O_2^-. Usually scavengers of O_2^- (SOD) and inhibitors of MPO activity (azide) are used to determine the relative importance of O_2^- and MPO-H_2O_2 in the metabolic response (31). The effects of SOD and azide in chemiluminescence systems are hard to evaluate (12), since, for example, SOD inhibits the chemiluminescence produced by a pure MPO-H_2O_2 system (12). To further analyze the role of MPO in the PMNL chemiluminescence response, we investigated the response of PMNL from an MPO-deficient donor. Cells from this donor have been studied twice, with essentially the same results.

MPO-deficient PMNL showed a pronounced decrease in the iodination of ingested yeast particles, but no reduced O_2^- production. Biochemical analysis of granule contents revealed a selective reduction of MPO (28). The observation that MPO-deficient PMNL produced almost no luminol-dependent chemiluminescence in response to fMLP, PMA, or opsonized yeast particles, despite a pronounced production of O_2^- (Table 1), indicated that luminol-dependent chemiluminescence was totally dependent on MPO. This agrees with the results recently published by DeChatelet et al. (13). The small amount of chemiluminescence observed in MPO-deficient PMNL after 120 min of preincubation may be related to a release of peroxidase from eosinophils, since these cells have been shown to contain peroxidase in MPO-deficient patients (21). Addition of purified MPO to the PMNL lacking this enyzme, before stimulus addition, resulted in a chemiluminescence response to all stimuli tested. In chemiluminescence systems without luminol, nonspecific augmentation of light emission as a result of protein addition has been described (16, 27). Oxidation of the added protein contributes directly to the chemiluminescence generated. This phenomenon does not influence the chemiluminescence in luminol-containing systems (17, 29), indicating that the effects of added MPO are enzymatically mediated.

When the time-course of fMLP-induced chemiluminescence emission from MPO-deficient PMNL supplemented with MPO was studied, only one peak, corresponding to the initial 2-min peak of control PMNL, was found (Fig. 1). The same type of time-course was obtained when control PMNL were exposed to fMLP in the presence of cyt B (Fig. 4). Since MPO-deficient PMNL in the presence of MPO gave rise only to an initial peak of chemiluminescence as a response to fMLP, and since the type of modulation of the response obtained when control PMNL were incubated at 22°C (Fig. 1 and 2) has been shown to be linked to the release of MPO during storage (12), we propose that the initial chemiluminescence peak observed is a result of extracellularly located MPO producing light by reacting with H_2O_2 released by the cells. Most of the cellularly produced H_2O_2 has O_2^- as its precursor and is formed by spontaneous dismu-

tation of O_2^-. When the time-course of chemiluminescence emission of MPO-deficient PMNL in the presence of MPO was studied, only an initial peak within 2 min was found after fMLP addition. However, depending on the preincubation time, the magnitude of the response differed (Fig. 1 and 2). Many of the receptors of chemotactic peptides are hidden and thus unavailable for ligand interaction (22). These receptors must be exposed (11), and degranulation could be one mechanism by which PMNL regulate the exposure of these receptors (15). Such a receptor development as a result of storage could explain the difference in magnitude of the chemiluminescence response to fMLP in the presence of MPO of MPO-deficient PMNL stored for different lengths of time at 22°C (Fig. 1 and 2).

Also, the second peak observed in luminol-dependent chemiluminescence in PMNL is linked to MPO, since no response was detected in MPO-deficient PMNL. The lack of second peak response to fMLP of the MPO-deficient PMNL in the presence of MPO could not be explained by consumption of the added MPO, since peak values of chemiluminescence of over 80,000 cpm were obtained with the same number of cells and only 0.25 μg of MPO per ml when PMA was used as stimulus (Fig. 3). This suggested that the presence of MPO in the extracellular fluid was not sufficient to obtain a normal chemiluminescence response from MPO-deficient PMNL. Upon interaction between PMNL and chemotactic peptides, the peptides are internalized by endocytosis (25, 30). It is possible that luminol may enter the cell and become oxidized intracellularly, unavailable to the extracellular MPO. Cyt B is known to affect release of oxidative metabolites and secretion of lysosomal enzymes, but also to inhibit PMNL endocytosis (20). The chemiluminescence obtained with control PMNL in the presence of cyt B (Fig. 4), taken together with the results obtained with the MPO-deficient PMNL, indicate that the second peak of activity could be a result of MPO reacting with H_2O_2 intracellularly after endocytosis of the peptide. This is further supported by earlier findings with catalase to inhibit the chemiluminescence in systems containing a larger number of PMNL (12). Catalase, a large-molecular-weight protein that may not have access to intracellular sites, was found to reduce the first peak of chemiluminescence, whereas the second peak was either unaffected or even increased (12).

In conclusion, the MPO-H_2O_2 system may not only be an important part of the mechanisms reponsible for microbicidal activity, but could also serve as an important modulator of the ceilular response to inflammatory mediators (4, 9, 12, 21, 31). Measurement of chemiluminescence could thus be a valuable and simple tool in studying MPO-H_2O_2 activity in inflammatory cells. However, in measuring chemiluminescence, caution must be taken when interpreting the cellular response to different agents, since many factors (stimuli, amount of MPO, release of MPO, production of H_2O_2) together determine both the magnitude and the time-course of the response.

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