Intra- and Extracellular Events 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. Luminol-dependent light emission from PMNL is linked to the myeloperoxidase (MPO)-
H$_2$O$_2$ system. Light emission from a cell-free MPO-H$_2$O$_2$ system was found to be totally inhibited by human serum albumin (HSA), and since HSA is a large molecular protein that does not readily gain access to
intracellular sites of PMNL, it could be used to determine the importance of extra- and intracellular events in
PMNL chemiluminescence. In studies with cells from an MPO-deficient patient, we found that HSA inhibited
more than 90% of extracellularly produced chemiluminescence. The chemotactic peptide formylmethionyl-leucyl-phenylalanine induced a two-peak chemiluminescence response in normal PMNL, and addition of HSA
reduced the first peak, whereas the second peak was unaffected. This result indicated that the first peak was
a result of extracellular reactions and the second peak was a result of intracellular reactions of the MPO-H$_2$O$_2$
system.

Most of the phorbol myristate acetate-induced response in normal PMNL was due to intracellular
events. Furthermore, chemiluminescence of intracellular origin seems to be limited not by generation of
oxidative metabolites but by diffusion of luminol into the cells.

The generation of chemically reactive molecules, e.g.,
superoxide anions, hydrogen peroxide, singlet oxygen, and
diroxyl radicals, as a result of respiratory burst activation
in polymorphonuclear leukocytes (PMNL) is essential for
host defense against microorganisms. Light emission or
chemiluminescence of PMNL, a phenomenon related to the
respiratory burst activation, was originally described by
Allen et al. (2). It has since been shown that the chemilumi-
nescence response of PMNL is influenced by a number of
factors (3, 9, 19) and that addition of 5-amino-2,3-dihydro-
1,4-phthalalenedione (luminol) to PMNL can amplify the
chemiluminescence response (1, 27). As a result of luminol
addition, the mechanism for light emission of PMNL
changes from involving both O$_2^-$ and myeloperoxidase
(MPO) (22) to being totally dependent on the MPO-H$_2$O$_2$
system (12, 13). Luminol acts as a bystander substrate for
the oxidative species generated during activation of the
PMNL, but it is not clear whether the oxidation takes place
extracellularly or whether luminol enters the PMNL and
becomes oxidized intracellularly (13, 16, 25). Since catalase,
also a large-molecular-weight scavenger for H$_2$O$_2$ that may not
have access to intracellular sites, reduces the luminol-
dependent chemiluminescence by only 50% (25) and since
addition of purified MPO to MPO-deficient PMNL has been
shown to only partially restore the response (12), it is
possible that some luminol may enter the PMNL and be
oxidized intracellularly.

The present investigation was designed to provide insight
into the role of extra- and intracellular events in luminol-
dependent chemiluminescence of human PMNL.

MATERIALS AND METHODS

PMNL. To obtain PMNL, EDTA-blood from normal
healthy laboratory personnel and from a previously de-
scribed MPO-deficient patient (9, 24) 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 in the same
buffer.

Reagents. Formylmethionyl-leucyl-phenylalanine (fMLP),
phorbol myristate acetate (PMA), and luminol were obtained
from the Sigma Chemical Co., St. Louis, Mo. Dimethyl
sulfoxide was used to dilute fMLP and PMA to 10$^{-2}$ M,
and water containing NaOH (0.1 M) was used to dissolve luminol
to 2×10$^{-2}$ M. The peptide, PMA, and luminol were further
diluted in KRG. Human serum albumin (HSA) was obtained
from AB Kabi, Stockholm, Sweden. Purified MPO from
human leukocytes was a generous gift from Inge Olsson
(Lund, Sweden).

Chemiluminescence measurements. Chemiluminescence
was measured at ambient room temperature in a modified
luminometer (LKB Instruments, Stockholm, Sweden). Samples
for chemiluminescence were obtained by adding 0.1 ml of
PMNL suspension or H$_2$O$_2$ diluted in KRG to disposable
4-ml polypropylene tubes containing 0.8 ml of KRG with: (i)
luminol (5×10$^{-7}$ to 5×10$^{-4}$ M), (ii) luminol-HSA, (iii)
luminol-MPO, or (iv) luminol-HSA-MPO. The tubes were
placed in the luminometer and allowed to stand until a stable
background was obtained (less than 1 min). To activate the
systems, 0.1 ml of fMLP, PMA, or MPO diluted to the
appropriate concentration in KRG was added. The tubes
were stirred, and the light emission was recorded continu-
ously. The experiments with normal PMNL were performed
at least three times, and the experiments with the MPO-
deficient PMNL were performed twice.

RESULTS

Effect of HSA on chemiluminescence in cell-free systems.
The MPO-H$_2$O$_2$-halide system is known to produce chemilu-
minescence in the absence of cells (22). The addition of MPO
to KRG containing H$_2$O$_2$ and luminol resulted in a pro-

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nounced chemiluminescence (Fig. 1). Light emission in MPO-H$_2$O$_2$ systems increased for the first 30 s after MPO addition and then declined. In the presence of HSA, no chemiluminescence was detected in MPO-H$_2$O$_2$ systems (Fig. 1). The chemiluminescence was totally depressed by HSA concentrations as low as 0.01% (data not shown).

**Effects of HSA on fMLP- and PMA-induced chemiluminescence.** The chemiluminescence response of PMNL is dependent on the MPO-H$_2$O$_2$ system, and since HSA has an inhibitory effect on light emission from the MPO-H$_2$O$_2$ system in the absence of cells and since HSA is a large molecular protein that may not have access to intracellular sites, the presence of HSA should primarily affect PMNL chemiluminescence generated extracellularly. When PMNL preincubated at 22°C for 60 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 at ca. 10 min after stimulus addition (Fig. 2). Also, in the presence of HSA, fMLP induced a two-peak response, but the initial 2-min peak was reduced to ca. 25% of its value in the absence of HSA, whereas the second 10-min peak was unaffected (Fig. 2). After the addition of PMA to the PMNL, there was a lag phase before any measurable signals were recorded. The peak activity was reached 20 to 25 min after stimulus addition. In the presence of HSA the initial rate was reduced, but the peak value was not changed (Fig. 3).

**Effect of HSA on light emission from MPO-deficient PMNL in the presence of MPO.** When MPO-deficient PMNL were exposed to PMA, almost no chemiluminescence response was observed. Addition of purified MPO to MPO-deficient PMNL before stimulus addition resulted in a chemiluminescence response when the cells were exposed to PMA (Fig. 4). The presence of HSA resulted in a pronounced reduction of the light emitted (Fig. 4).

**Effect of various concentrations of luminol on fMLP- and PMA-induced chemiluminescence.** The intensity of the fMLP- (Fig. 5) and PMA-induced (Fig. 6) chemiluminescence increased when the luminol concentration was increased. Also, the time-course changed as a result of increased luminol concentration. The peak activity in the PMA-induced response was reached earlier at low luminol concentrations. In the fMLP-induced response, initially both the first and the second peak were increased by increased luminol concentration, but at higher concentrations, a saturation of the first peak value was observed (Fig. 7). The saturation phenomenon observed for the first peak in the fMLP-induced response was not observed for the second peak or for the PMA-induced response.

**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 it 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. Usually scavengers, e.g., superoxide dismutase and catalase, or inhibitors, e.g., azide, are used to determine the relative importance of the reactive molecules in the metabolic response (28), but in chemiluminescence systems, the effects of these substances are difficult to evaluate (11). Chemiluminescence data obtained with cells from patients with myeloperoxidase deficiencies have indicated that the luminol-dependent chemiluminescence is totally dependent on the MPO-H$_2$O$_2$ system (12, 13, 25).
Most of the cellularly produced H$_2$O$_2$ has O$_2^-$ as its precursor and is formed by spontaneous dismutation of O$_2^-$ (21), and O$_2^-$ is also produced by MPO-deficient cells (22). The presence of MPO in the extracellular fluid is, however, not sufficient to obtain a normal chemiluminescence response from MPO-deficient PMNL (12), indicating that the reaction leading to luminol-dependent light emission from PMNL takes place both intra- and extracellularly.

In luminol-enhanced chemiluminescence systems, the oxidation of luminol generates an excited aminophthalate anion that relaxes to the ground state with the production of light; however, the exact reaction leading to the excited state is not known (13). In cell-free MPO-H$_2$O$_2$ systems, we found that HSA in concentrations between 0.01 and 1% totally inhibited light emission (Fig. 1). Since the light-producing reaction is not defined, the mechanism for inhibition with HSA is unknown. Bulk movement of extracellular fluid (pinocytosis) into PMNL does not occur to any appreciable extent (8, 10), and since human PMNL lack receptors for HSA (29), this large molecular protein does not readily gain access to intracellular sites of PMNL, indicating that HSA could possibly be used to determine the importance of extra- and intracellular events in PMNL luminol-dependent chemiluminescence.

FIG. 4. Time trace of chemiluminescence emitted from MPO-deficient PMNL (2 × 10$^6$/ml) with added purified MPO (1 µg/ml) when exposed to PMA (10$^{-7}$ M) in the absence (solid line) and presence (dashed line) of HSA (1% [wt/vol]). Abscissa, time of study (min); ordinate, chemiluminescence (mV).

FIG. 5. Time trace of chemiluminescence at different luminol concentrations (5 × 10$^{-7}$ to 5 × 10$^{-5}$ M) emitted from normal human PMNL (2 × 10$^6$/ml) incubated at 22°C for 120 min when exposed to fMLP (10$^{-7}$ M). Abscissa, time of study (min); ordinate, chemiluminescence (mV).

FIG. 6. Time trace of chemiluminescence in different luminol concentrations (10$^{-6}$ to 10$^{-4}$ M) emitted from normal human PMNL (2 × 10$^6$/ml) when exposed to PMA (10$^{-7}$ M). Abscissa, time of study (min); ordinate, chemiluminescence (mV).

FIG. 7. Chemiluminescence at different luminol concentrations emitted from normal human PMNL (2 × 10$^6$/ml) when exposed to fMLP (10$^{-7}$ M). Abscissa, luminol concentration (M); ordinate, chemiluminescence peak value (mV) of the initial (2-min) peak (△) and the second (10-min) peak (●).
When PMNL 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 at ca. 10 min after stimulus addition (Fig. 2). The addition of HSA had a pronounced effect on the response in that the initial peak of chemiluminescence was reduced, whereas the second peak was unaffected (Fig. 2), indicating that the initial peak is a result of extracellular and the second peak is a result of intracellular reactions of the MPO-H₂O₂ system. This is further supported by earlier findings with catalase, another large-molecular-weight protein that was found to reduce the first peak of chemiluminescence, whereas the second peak was either unaffected or even increased (11), and those with MPO-deficient PMNL in the presence of MPO, in which only an initial peak of chemiluminescence was observed (12). The second peak could be a result of endocytosis of the peptide. On interaction between PMNL and chemotactic peptides, the peptides are internalized by endocytosis (18, 20, 23, 26), and it is possible that luminol may enter the cells and become oxidized intracellularly, being unable to gain access to the extracellular environment. This agrees with the recently published results of Bender and van Epps (6).

After addition of PMA to the PMNL there was a lag phase before any measurable signals were recorded. The length of the lag phase in a PMA response has been shown to depend on the concentration of the stimulus (14). The peak activity as a response to PMA was reached after 20 to 25 min. The peak activity was unaffected by addition of protein but the initial rate was decreased, indicating that extracellular reactions of the MPO-H₂O₂ system are of importance early in the reaction between PMNL and PMA, whereas intracellular reactions predominate later on in the response. The experiments have been repeated four times with essentially the same result. In chemiluminescence systems without luminol, nonspecific augmentation of light emission as a result of protein addition has been described (15, 22). Oxidation of the added protein contributes directly to the chemiluminescence generated, a phenomenon not observed in PMNL chemiluminescence in luminol-containing systems (16, 25).

To further analyze the effects of HSA on PMNL chemiluminescence response, we investigated the effect of HSA on the response of PMNL from an MPO-deficient donor. The observation that MPO-deficient PMNL required addition of MPO in the extracellular fluid to produce chemiluminescence, despite a pronounced production of O₂⁻ (12), indicates that the chemiluminescence of these cells in the presence of purified MPO has an extracellular origin. Furthermore, in the response to PMA of PMNL from the MPO-deficient donor (in the presence of MPO), the peak activity was reached much earlier than in the response of normal PMNL (5 to 10 min compared with 25 to 30 min). This further supports the conclusion that extracellular reactions are of importance early in the PMA-induced response. The presence of HSA resulted in a pronounced decrease in the light emitted from MPO-deficient PMNL with added MPO. From the peak values of the response obtained in the absence and in the presence of HSA, it could be calculated that the extracellularly generated chemiluminescence was reduced by more than 90% in the presence of HSA.

The fact that increased concentrations of luminol primarily enhanced the second peak of the fMLP and the PMA response indicates that the intracellularly generated chemiluminescence may be limited by the diffusion of luminol into the cell. However, the ratio of luminol/H₂O₂ may also directly affect the time-course of the light emitted from a cell-free peroxidase-H₂O₂-luminol system (17).

In conclusion, measurement of luminol-enhanced chemiluminescence could be a valuable and simple tool in studying metabolic activity in inflammatory cells. However, in measuring chemiluminescence, caution must be taken when interpreting the cellular response to different agents since many factors, such as stimulus, amount of MPO, release of MPO, and production of H₂O₂, together determine both the magnitude and the time-course of the response. Furthermore, since luminol seems to diffuse into phagocytic cells, this diffusion may create a problem in quantitative measurements of chemiluminescence in that the reaction may be limited not by generation of oxidative metabolites but by the diffusion of luminol into the cells.

**LITERATURE CITED**


