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Effect of Chemoattractants on Chemiluminescence

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Upon ingestion of particulate matter, polymorphonuclear leukocytes produce a chemiluminescence that can be measured in a liquid scintillation counter. In the experiments reported here, the influence of three chemoattractants and three chemotactic modulators upon the chemiluminescence induced by opsonized zymosan was studied. The chemoattractants investigated (including bacterial factor derived from *Escherichia coli*, the simple peptide formylmethionylalanine, and activated human complement), which initiate directed movement when presented to cells in a concentration gradient, significantly enhanced zymosan-induced chemiluminescence. In the absence of opsonized zymosan, however, they had no effect on the chemiluminescence response. In contrast, the chemotactic modulators studied (including carbamylcholine, phenylephrine, and cyclic guanosine 5'-monophosphate, which are not chemotactic by themselves but can enhance or depress the movement of polymorphonuclear leukocytes initiated by chemoattractants) produced no enhancement of chemiluminescence. Other experiments were carried out in which neutrophils were pretreated with cytochalasin D, a compound that inhibits phagocytosis by interacting with microfilaments. Under these conditions, the chemiluminescence induced by opsonized zymosan was markedly reduced, but the response resulting from the addition of a chemoattractant to the leukocyte/zymosan mixture was not. This indicates that the chemiluminescence in response to chemoattractants is not dependent on phagocytosis per se. Neutrophils were also pretreated with dinitrofluorobenzene, a compound that binds amino groups and can be expected to react with proteins on the cell membrane. In these experiments, the chemiluminescence induced by opsonized zymosan and the pronounced spike of activity produced by the addition of a chemoattractant were completely abolished. These results suggest that the polymorphonuclear leukocyte chemiluminescence response to chemoattractants is mediated by cell surface proteins. Thus, chemoattractants may have a dual role in the acute inflammatory response: (i) the initiation and maintenance of directed cell movement, and (ii) enhancement of metabolic steps mediated at the cell membrane, resulting in microbicidal activity.

The acute inflammatory response to a variety of offending microbes can be simplistically characterized as the sequential occurrence of the chemotactic, phagocytic, and microbicidal processes. Many compounds, referred to here as chemoattractants, are known to exist which influence the chemotactic phase of the inflammatory response. When these substances are presented to leukocytes in a concentration gradient, they can initiate and maintain directed cell movement. In contrast, compounds referred to here as chemotactic modulators are known which do not initiate cell movement by themselves but can enhance or depress motion induced by chemoattractants (5, 7). Since both chemoattractants and chemotactic modulators profoundly affect chemotaxis, a function controlled in part at the cell membrane, it seemed reasonable to speculate that these same compounds might influence other aspects of the inflammatory response requiring cell membrane participation such as phagocytosis or microbicidal activity. To investigate this possibility, the chemiluminescence response of neutrophils induced by opsonized zymosan was selected for study. Although the exact mechanism of chemiluminescence production is controversial, it has been suggested that, upon contact with or ingestion of particulate matter, the microbicidal mechanism of the neutrophil is activated, and excited molecular oxygen and carbonyl groups are generated (1, 2). Decay to the ground state of these excited molecular forms results in a chemiluminescence that can be detected and quantitated in a liquid

scintillation counter. Based on these assumptions, the chemiluminescence measured may reflect the level of phagocytic and/or microbicidal activity of the cell. The present studies indicate that chemoattractants, but not chemotactic modulators, enhance the chemiluminescence response of polymorphonuclear leukocytes (PMNs) to opsonized zymosan. This enhanced response was not blocked by inhibition of phagocytosis with cytochalasin D but was abolished by binding cell surface proteins with dinitrofluorobenzene. Thus, the chemiluminescence response appears to be mediated by cell membrane proteins that are affected by chemoattractants.

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MATERIALS AND METHODS

Leukocyte preparations. Heparinized human blood (10 U/ml) from healthy donors was allowed to settle for 30 to 60 min at 37°C. The leukocyte-rich plasma on top was then removed with a syringe, and the cells were sedimented from this suspension by centrifugation at 100 × *g* for 10 min. The pellet was suspended and washed twice in sterile phosphate-buffered saline (PBS, 4,500 ml of distilled water, 5.2 g of Na_2HPO_4, 0.9 g of KCl, 0.9 EM KH_2PO_4, and 36 g of NaCl). The washed cells were counted on a hemacytometer and adjusted to a concentration of 1.0×10^7 PMNs per ml in PBS.

Opsonization of zymosan. Zymosan particles (100 mg) were washed once in 10 ml of PBS, and the pellet was suspended in donor serum to a concentration of 10 mg/ml in a sterile plastic tube as previously described (8). The suspension was mixed in a Vortex mixer, capped, and rotated on a Roto rack (Fisher Scientific, Santa Clara, Calif.) for 60 min at 37°C. The suspension was then centrifuged, the serum was decanted, and the pellet was resuspended in PBS to a concentration of 10 mg/ml. The decanted serum was saved as a source of the chemotactically active complement fragments resulting from interaction of the serum with zymosan.

Chemiluminescence procedure. The chemiluminescence procedure was performed as previously described (6, 8). Control reaction mixtures contained 2.8 ml of PBS, 0.2 ml of opsonized zymosan (10 mg/ml), and 0.5 ml of the cell suspension (5×10^6 PMNs) for a total volume of 3.5 ml. The test reaction mixtures contained 2.45 ml of PBS, 0.35 ml of test agent, 0.2 ml of opsonized zymosan (10 mg/ml), and 0.5 ml of the cell suspension (5×10^6 PMNs) for a final volume of 3.5 ml. The mixtures were made in Beckman (Fullerton, Calif.) Poly Q scintillation vials which were wrapped in aluminum foil and stored in the dark for at least 24 h before use. The vial was immediately capped upon introduction of the reaction mixture and placed in a Beckman (Fullerton, Calif.) LS-100c scintillation counter, out of phase, with one photomultiplier tube disconnected. Duplicate vials for each test condition were counted at approximately 10-min intervals for 60 to 90 min and the results were recorded in mean counts per minute.

Pretreatment of leukocytes. In pretreatment experiments, the test agents were added to cells already prepared as discussed above. Cytochalasin D (Aldrich Chemical, Milwaukee, Wis.) in PBS solution was added to the cell suspension to a final concentration of 10 μg/ml, and dinitrofluorobenzene in PBS solution was added to a final concentration of 2.5×10^{-2} M. These solutions were rotated on a Roto rack for 10 min at 37°C. The cells were centrifuged, washed once in PBS, and resuspended to original volume and concentration of 1.0×10^7 PMNs per ml in PBS. The viability of pretreated cells was compared with controls by determining the extent of 0.01% trypan blue uptake.

Reagents. Phenylephrine, formylmethionylalanine, carbachol, glutamic acid, triolein, and cyclic guanosine 5′-monophosphate were obtained from Sigma, St. Louis, Mo. Zymosan was obtained from Schwartz/Mann, Orangeburg, N.Y. Phorbol myristate acetate was obtained from Consolidated Midland, Brewster, N.Y.

Escherichia coli bacterial factor was prepared from an overnight growth of *E. coli* in medium 199 (16). A group B streptococcal bacterial chemotactic factor was prepared in a similar manner from a type III group B strain isolated from a newborn infant with sepsis.

Cytochalasin D was initially dissolved in dimethyl sulfoxide to a concentration of 10^{-2} M and then diluted in PBS to the final concentration used in the experiments. This represented a 500× dilution of dimethyl sulfoxide in PBS; control experiments showed that this concentration did not influence chemiluminescence.

RESULTS

The results of an experiment carried out with the *E. coli* chemotactic factor are shown in Fig. 1. The addition of the *E. coli* bacterial factor to the reaction mixture significantly enhanced PMN chemiluminescence induced by opsonized zymosan. A similar enhancement was produced by a group B streptococcal culture filtrate (data not shown). The peak in chemiluminescence increased in most experiments by 5,000 to 20,000 cpm when the streptococcal or *E. coli* bacterial factor was present throughout the experiment. Similar results (data not shown) were observed when the PMNs were preincubated in the bacterial factor, washed, and then used in the procedure. This implies that the chemoattractants interact with the leukocytes rather than with the zymosan to enhance chemiluminescence. No significant effect on chemiluminescence was observed when bacterial factor was added to cells alone or to the zymosan alone before the addition of leukocytes. A slight enhancement of chemiluminescence was observed when bacterial factor was added to PMNs and unopsonized zymosan.

Because the bacterial factor has not been com-

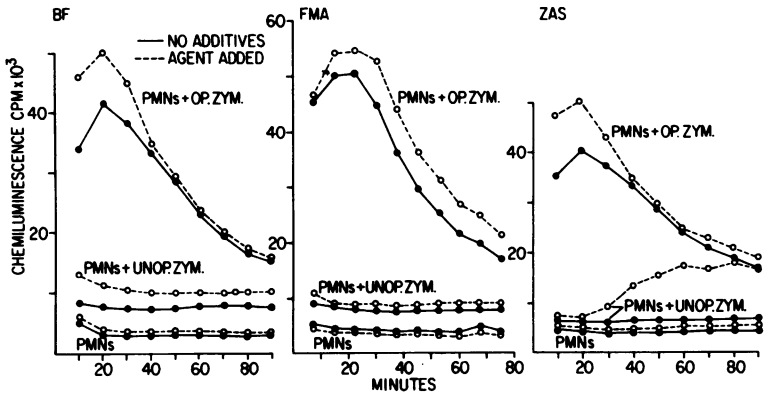

FIG. 1. *Experiments were carried out in which the chemoattractants were present in the reaction mixture throughout the experiment. Each chemoattractant (10% bacterial factor [BF], 10^{-4} M formylmethionylalanine [FMA], or 10% zymosan-activated serum [ZAS]) significantly enhanced the chemiluminescence response to opsonized zymosan. No effect was observed when the chemoattractants were added to cells alone. A slight enhancement of chemiluminescence was observed when bacterial factor and formylmethionylalanine were added to PMNs and unopsonized zymosan. When zymosan-activated serum was added to the reaction mixture containing PMNs and unopsonized zymosan, it was apparent that some opsonization occurred after 20 to 30 min of incubation.*

pletely characterized, a more basic compound was sought for additional studies. Schiffman and co-workers (14) have indicated that the formylmethionyl peptides possess significant chemotactic activity. Formylmethionylalanine was, therefore, investigated for its effect on chemiluminescence. In a manner similar to bacterial factor, formylmethionylalanine significantly enhanced the chemiluminescence response induced by opsonized zymosan (Fig. 1). Again, no effect was observed on cells alone. Additional experiments demonstrated that the non-chemotactically active amino acid methionine did not enhance the chemiluminescence response (data not shown). This demonstrates that the results with the chemoattractants are not due to interaction with proteinaceous material per se. Also considered was the possibility that the hydrophilic nature of the chemoattractants tested was responsible for the enhancement of chemiluminescence observed. Experiments performed with glutamate, an amino acid with very limited aqueous solubility, and triolein, a medium-chain triglyceride, showed no enhancement of chemiluminescence.

Serum activated with zymosan to release chemotactically activated complement fragments also enhanced the chemiluminescence response to opsonized zymosan (Fig. 1). When the zymosan-activated serum was added to a reaction mixture containing PMNs and unopsonized zymosan, it was apparent that some opsonization occurred. There was no effect of zymosan-activated serum on cells alone, however.

Pulse experiments with the chemoattractants were carried out, in which the agents were added to the reaction mixture approximately 30 min after initiation of the procedure (Fig. 2). Each chemoattractant significantly enhanced the chemiluminescence response to opsonized zymosan. The control cells were pulsed with PBS and demonstrated no enhancement of chemiluminescence in response to light exposure during the pulsing procedure.

Additional experiments were performed with the three enhancing chemotactic modulators to determine their effects on chemiluminescence (data not shown). Phenylephrine, carbamylcholine, and cyclic guanosine 5′-monophosphate had no effect on the chemiluminescence response. Interestingly, phorbol myristate acetate, which is an enhancing chemotactic modulator (7), showed activity characteristic of a chemoattractant in the chemiluminescence assay (Fig. 3). This agent greatly enhanced chemiluminescence induced by opsonized zymosan but did not significantly increase the luminescence of cells alone at the concentrations tested (≤10 ng/ml). DeChatelet et al. have demonstrated, and we have confirmed, that phorbol myristate acetate enhances chemiluminescence in resting leukocytes (3) with concentrations of the drug 10 to 1,000-fold higher than utilized in the experiments reported here. Lower concentrations were investiated in this lab because they had been previously shown to optimally enhance chemotaxis and intracellular cyclic guanosine 5′-monophosphate accumulation (5, 7).

In an attempt to delineate further the nature of the chemiluminescence enhancement produced by the chemoattractants, experiments were conducted in which PMNs were pretreated

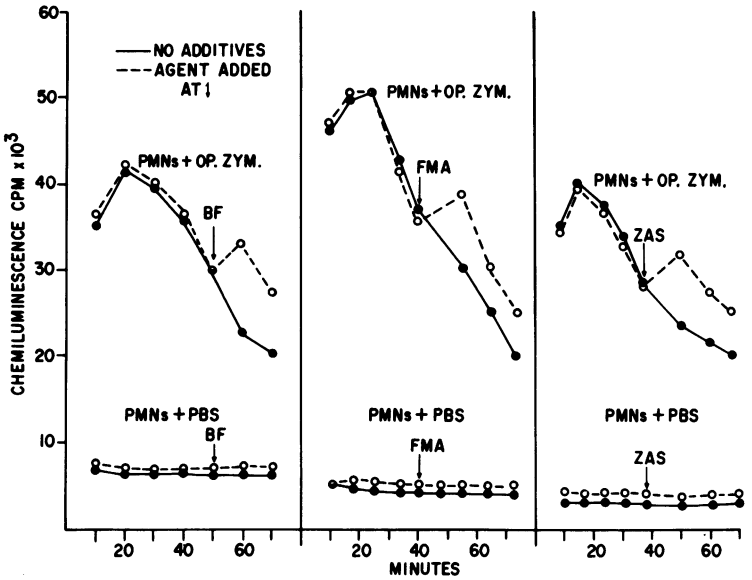

FIG. 2. *Pulse experiments with the chemoattractants were carried out in which the agents were added to the reaction mixture approximately 30 min after initiation of the procedure. Each chemoattractant (10% bacterial factor [BF], 10^{-4} M formylmethionylalanine [FMA], or 10% zymosan-activated serum [ZAS]) significantly enhanced the chemiluminescence response to opsonized zymosan.*

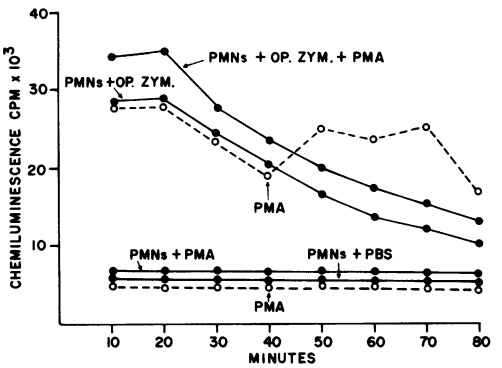

FIG. 3. *Phorbol myristate acetate (PMA), which is an enhancing chemotactic modulator, showed activity characteristic of a chemoattractant by significantly enhancing the chemiluminescence response to opsonized zymosan (at a concentration of 10 ng/ml).*

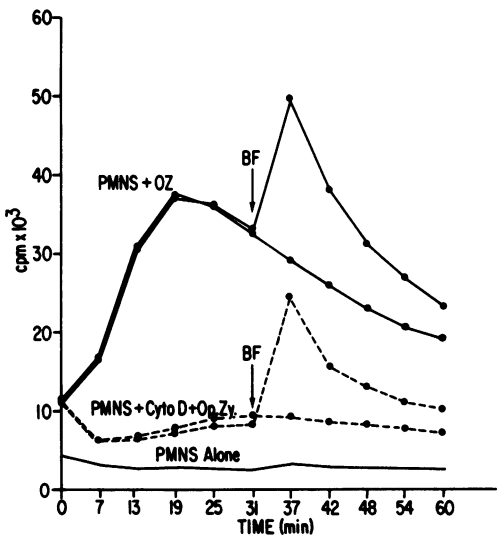

FIG. 4. *Chemiluminescence induced by opsonized zymosan was markedly inhibited by pretreating PMNs with cytochalasin D (10 μg/ml). The enhancement of chemiluminescence produced by a pulse of bacterial factor, in contrast, was not blocked by cytochalasin.*

with cytochalasin D. This compound is thought to inhibit phagocytosis by interacting with microfilaments (12). Figure 4 demonstrates that the chemiluminescence induced by opsonized zymosan was markedly inhibited by cytochalasin D at a concentration that almost completely prevented particle uptake as determined visually. The enhancement of chemiluminescence produced by a pulse of bacterial factor, in contrast, was not blocked by cytochalasin. This result suggests that the chemoattractants are acting independently of phagocytosis per se.

Figure 5 shows the results obtained when cells were pretreated with dinitrofluorobenzene. This compound has been shown to nonspecifically bind amino groups (11) and can be expected to react with the cell surface membrane. As shown,

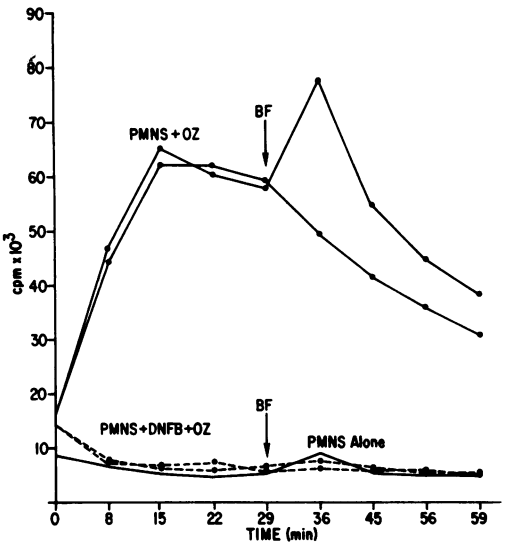

FIG. 5. *Cells pretreated with 2.5 × 10^{-2} M dinitrofluorobenzene (DNFB) demonstrated complete inhibition of chemiluminescence induced by opsonized zymosan or a pulse of bacterial factor.*

dinitrofluorobenzene prevents chemiluminescence induced by opsonized zymosan and the chemiluminescence enhancement resulting from bacterial factor. Phagocytosis in the cells pretreated with dinitrofluorobenzene was not prevented, since ingested particles could be visually demonstrated within the cells. The viability of the pretreated cells compared with controls was similar as shown by a lack of trypan blue uptake in either cell population.

DISCUSSION

Although the biochemical mechanism of microbicidal activity in neutrophils is presently incompletely elucidated, probably the most widely held view is that killing of organisms depends upon oxidative metabolism associated with phagocytosis (1, 9). During particle ingestion, oxygen uptake and hexose monophosphate shunt activity dramatically increase, and superoxide anion (O_2^-) is generated. This excited form of molecular oxygen reacts to produce the potent microbicidal oxidants hydrogen peroxide (H_2O_2) and hydroxyl radical (OH) or may spontaneously dismutate to a less energetic singlet form of oxygen. It is postulated that the occurrence of this last reaction in neutrophils is associated with the emission of photons and is likely responsible for the luminescence accompanying phagocytosis. Experiments reported in this paper demonstrate that chemoattractants, which are capable of initiating the chemotactic response, significantly enhance the chemiluminescence produced by PMNs in the presence of ingestible particles. In contrast, the enhancing chemotactic modulators do not augment neutrophil chemiluminescence.

Recently, Musson and Becker (13) have demonstrated that chemoattractants decrease the initial rate of phagocytosis of antibody and complement-coated sheep erythrocytes (EAC) by PMNs. Such treatment did not appear to alter attachment of the EAC1423 complex to the PMNs or to affect overall levels of uptake at 90 min. Furthermore, the inhibition of phagocytosis induced by chemotactic factors could be overcome by increasing the concentration of ingestible particles used in the procedure. Using somewhat higher concentrations of similar chemoattractants (10- to 100-fold higher) and an increased number of particles per PMN (20-fold higher), we found that chemoattractants significantly enhance chemiluminescence. Furthermore, this stimulation of chemiluminescence appeared to be mediated at the cell surface rather than by phagocytosis per se. Chemoattractants are known to interact with the cell surface during the process of initiating chemotaxis. It has been demonstrated that treatment of peripheral blood leukocytes with chemoattractants results in the acquisition of membrane-bound esterase activity and chemotactic deactivation (15). Evidence such as this, which demonstrates that chemoattractants influence chemotaxis by a membrane interaction, motivated the experiments here aimed at learning whether chemoattractants might also influence chemiluminescence by a membrane interaction. Experiments presented in this paper indicate that cytochalasin D prevented phagocytosis of opsonized zymosan particles and profoundly inhibited chemiluminescence production. The enhancement of chemiluminescence produced by a pulse of the chemoattractant bacterial factor was not inhibited, however. Similar results were obtained with colchicine (1 to 10 mM), a drug that inhibits phagocytosis probably through an action on microtubules (data not shown). This evidence suggests that chemiluminescence is mediated in part at the membrane and that the chemiluminescence enhancement by bacterial factor is not dependent on phagocytosis. These findings are similar to those of Johnston and colleagues (10), who found that surface acting agents as well as surface-bound immunoglobulin G triggered monocyte chemiluminescence. In our experiments, it was also shown that cells pretreated with dinitrofluorobenzene demonstrated complete inhibition of chemiluminescence induced by opsonized zymosan and the enhancement of chemiluminescence induced by a pulse of the chemoattractant bacterial factor. This compound bound

strongly to proteins but did not prevent phagocytic uptake. These experiments suggest, therefore, that chemiluminescence induced by opsonized zymosan or the bacterial factor is initiated through an interaction with protein receptors. Goldstein and others (4) reported similar results by showing that neutrophils exposed to *p*-diazobenzene sulfonic acid (a reagent that predominantly reacts with membrane proteins) demonstrated significantly decreased O_2^- generation in response to lectin, which reacts with cell surfaces.

In summary, our results demonstrate that chemoattractants enhance chemiluminescence induced by opsonized zymosan and that this enhancement is not dependent upon actual particle uptake. Our results support the possibility that chemiluminescence is mediated by membrane-bound proteins. We can only speculate about the potential importance of this phenomenon in vivo. Release of chemoattractants at the site of microbial invasion or injury and subsequent diffusion sets up a chemotactic gradient in the area. This gradient results in an influx of phagocytes. As suggested by Musson and Becker (13), low concentrations of the chemoattractants at the cell surface during this phase of cell migration may actually inhibit phagocytosis. Upon reaching the site of microbial invasion, however, the cells are presented with a large number of ingestible particles which may help to overcome the inhibitory influence on phagocytosis. In addition, they are surrounded by a higher concentration of chemoattractant, which results in maximal stimulation of the metabolic activity of these inflammatory cells. This could result in (i) an enhanced microbicidal capacity, or (ii) release of toxic products of oxidation into the surrounding area, which could result in increased tissue damage.

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