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Chemiluminescent Response of Polymorphonuclear Leukocytes to *Streptococcus pneumoniae* and *Haemophilus influenzae* in Suspension and Adhered to Glass

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We measured the luminol- and lucigenin-enhanced chemiluminescent response of human polymorphonuclear leukocytes (PMN) stimulated by various strains of *Streptococcus pneumoniae* and *Haemophilus influenzae*. In the absence of opsonin, phagocytosis of either bacterial species elicited good PMN response when the bacteria were adhered to a surface but minimal PMN response when they were in suspension. When 10% pooled human serum was used as a source of opsonin, a moderate to excellent chemiluminescent PMN response was elicited during phagocytosis of opsonized bacteria both in suspension and adhered to a surface. We conclude that opsonin significantly enhances PMN chemiluminescence when a suspension-type assay is used and that opsonin-independent mechanisms play a significant role in the chemiluminescent response of PMN during phagocytosis of adherent bacteria.

Previous studies have shown that certain strains of *Streptococcus pneumoniae* are ingested by polymorphonuclear leukocytes (PMN) in the absence of opsonins (15, 17). This phenomenon generally occurs when the bacteria are trapped or held against a hard surface. A recent study has shown that PMN and alveolar macrophages can use an opsonin-independent mechanism of phagocytosis when they encounter bacteria adhered to a surface (8, 9). This opsonin-independent surface phagocytosis may play an important role in host defense against respiratory tract infections.

S. pneumoniae and *Haemophilus influenzae* are two common respiratory pathogens. Both species are encapsulated, and resistance to phagocytosis has been attributed predominantly to the presence of a capsule (12, 16).

The purpose of this investigation was to examine the interaction of these bacterial species with human PMN for bacteria in suspension and adhered to a surface. The ability to elicit a chemiluminescent response from PMN was assayed because chemiluminescence measures the oxidative metabolic burst of PMN in response to membrane perturbation. The encapsulated strains *S. pneumoniae* 7F and *H. influenzae* type b were compared with the unencapsulated strains *S. pneumoniae* R36A and *H. influenzae* non-type b. PMN chemiluminescence was compared between the suspension and surface-adhered phagocytosis assays. The effect of complement opsonin on the chemiluminescent response of PMN to these strains was also compared between the two phagocytosis assays.

MATERIALS AND METHODS

Preparation of bacteria. *S. pneumoniae* 7F and R36A were grown in 10 ml of Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) for 18 h at 37°C in a shaking incubator. *H. influenzae* type b and non-type b were grown in 10 ml of Todd-Hewitt broth supplemented with hemin type III and NAD (10 μg of each per ml; Sigma Chemical Co., St. Louis, Mo.). These strains were also grown for 18 h at 37°C in a shaking incubator. All strains were centrifuged three times, washed twice with phosphate-buffered saline, and suspended to a concentration of 10^9 CFU/ml of phosphate-buffered saline by a spectrophotometric method.

PMN preparation. Peripheral venous blood was collected from healthy adult volunteers into a heparinized syringe. PMN were separated from whole blood by a previously described method (3, 13). The final PMN suspension contained 10^5 cells/ml of Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.) containing 0.1% gelatin (GHBSS). The purity of the PMN suspension and cell viability as determined by trypan blue exclusion exceeded 95%.

Preparation of chemilumogenic probes. Luminol (5-amino-2,3-dehydro-1,4-phthalazinedione) (Eastman Kodak Co., Rochester, N.Y.) was prepared as a 275 μM stock solution in dimethyl sulfoxide (Sigma). Luminol stock solution was diluted in GHBSS to a final concentration of 1 μM per vial.

Lucigenin (bis-*N*-methylacridinium nitrate) (Sigma) was prepared as a 6.88 mM stock solution in sterile water. Lucigenin stock solution was diluted in GHBSS to a final concentration of 100 μM per vial.

Chemiluminescence assay. The chemiluminescence assay was performed by a modification of previously reported microchemiluminescence techniques (1, 2, 8, 10). To examine the chemiluminescent response of PMN to bacteria in suspension, 0.8 ml of a luminol or lucigenin solution was added to a glass vial (12 mm diameter) with 0.1 ml of a bacterial suspension (10^7 CFU). The vials were then placed in a Picolite luminometer (Packard Instrument Co., Downer's Grove, Ill.) maintained at 37°C and mixed by a magnetic stirring mechanism at 240 rpm to ensure suspension. Bacteria under suspension conditions were opsonized by adding 0.1 ml of bacteria (10^9 CFU/ml), 0.1 ml of pooled human serum (PHS) frozen at −70°C, and 0.8 ml of GHBSS. Tubes were mixed at 10 rpm on a rotating rack at 37°C for 15 min. This mixture was centrifuged at 3,000 × *g* for 15 min, and the bacterial pellet was suspended to a final concentration of 10^8 CFU/ml in GHBSS.

Surface chemiluminescence conditions were obtained by adding 0.8 ml of a luminol or lucigenin solution to a vial

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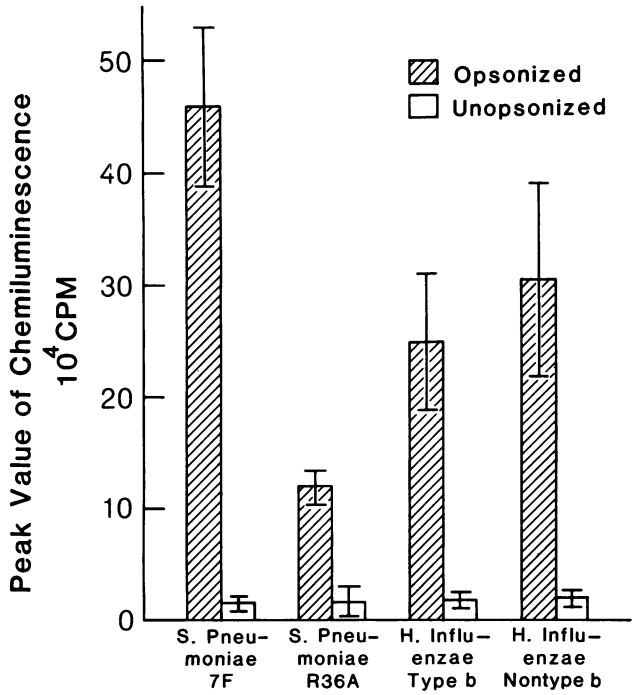

FIG. 1. Peak values of luminol-enhanced suspension chemiluminescence (10^4 cpm/10^4 PMN). Opsonized bacteria were pretreated with 10% PHS. Bars indicate the standard error of the mean (SEM).

containing 10^7 adherent bacteria and 0.1 ml of GHBSS. If opsonized bacteria were desired, 0.2 ml of 10% PHS was added to the vial, which was then incubated for 15 min at 37°C in a stationary position. After this incubation, the supernatant was discarded. These vials were also placed in the luminometer but were not mixed.

Background counts per minute were obtained for all vials before 0.1 ml of a PMN suspension (10^4 cells) was automatically injected. A final ratio of 1 PMN to 1,000 bacteria was used in each vial. The chemiluminescent response of each vial was measured for 10 s at 5-min intervals for 60 min. The data were analyzed for statistical significance by Student's *t* test.

RESULTS

The luminol-enhanced chemiluminescent response of granulocytes to opsonized bacteria in suspension is shown in Fig. 1. The luminol-enhanced chemiluminescent response of PMN to nonopsonized bacteria in suspension was equal to that of background PMN suspended in GHBSS only. The luminol-enhanced chemiluminescent response of PMN was higher during phagocytosis of *S. pneumoniae* 7F (an encapsulated strain) than during phagocytosis of *S. pneumoniae* R36A (an unencapsulated strain) ($P < 0.01$). The luminol-enhanced chemiluminescent response of PMN to *H. influenzae* type b was equal to the response to *H. influenzae* non-type b for opsonized bacteria in suspension (Fig. 1). Chemiluminescence was equal to background for nonopsonized *H. influenzae* in suspension.

When *S. pneumoniae* 7F and R36A were adhered to glass, a moderate chemiluminescent response was elicited in both the presence and absence of opsonin (Fig. 2). When *H. influenzae* type b and non-type b were adhered to glass, a PMN chemiluminescent response was elicited in both the presence and absence of opsonin. The PMN chemiluminescent response to opsonized *S. pneumoniae* and *H. influenzae* in suspension was approximately two times higher than the response to opsonized bacteria adhered to glass.

The lucigenin-enhanced PMN chemiluminescent response to opsonized *S. pneumoniae* R36A in suspension was higher than that to opsonized *S. pneumoniae* 7F (Fig. 3). This is the reverse of what was observed when luminol-enhanced chemiluminescence was compared ($P < 0.01$ for both). A similar chemiluminescent response was elicited with type b and non-type b *H. influenzae* (Fig. 3).

The lucigenin-enhanced PMN chemiluminescence elicited by opsonized *S. pneumoniae* 7F was equal to that by opsonized *S. pneumoniae* R36A when both were adhered to a surface (Fig. 4). This result was also observed when *H. influenzae* type b was compared with non-type b under similar conditions (Fig. 4). Unopsonized bacteria in suspension did not elicit a chemiluminescent response, but adherent unopsonized bacteria elicited a moderate to good response with both luminol and lucigenin.

DISCUSSION

Recent studies in our laboratory have shown that PMN and alveolar macrophages can utilize opsonin-independent mechanisms of phagocytosis when bacteria are adhered to a solid surface (8, 9). A chemiluminescent response can be measured when PMN are incubated with unopsonized *S. aureus* and *P. aeruginosa* adhered to glass, plastic, or agar (8). Both luminol- and lucigenin-enhanced chemiluminescence assays were used to investigate the response of PMN to opsonized and unopsonized *S. pneumoniae* and *H. influenzae* in suspension and adhered to a surface. Luminol-enhanced chemiluminescence results primarily from myeloperoxidase (MPO)-dependent production of H_2O_2, and lucinogen-enhanced chemiluminescence records PMN superoxide production (4–6).

Opsonin-independent PMN chemiluminescence was elic-

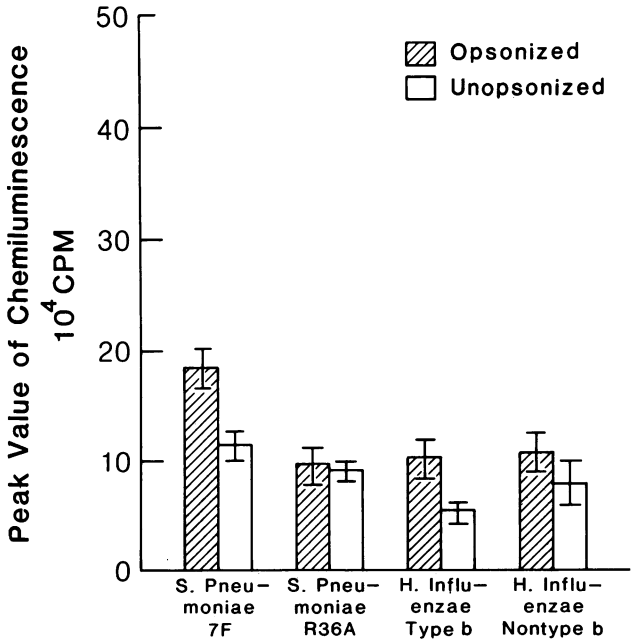

FIG. 2. Peak values of luminol-enhanced surface chemiluminescence (10^4 cpm/10^4 PMN). Opsonized bacteria were pretreated with 10% PHS. Bars indicate SEM.

ited by both encapsulated and nonencapsulated *S. pneumoniae* and *H. influenzae* strains. PMN chemiluminescence, therefore, was influenced very little by the presence or absence of capsular material when bacteria were adhered to glass surfaces. Both luminol- and lucigenin-dependent PMN chemiluminescence was magnified, however, when opsonin was added. This was most striking when PMN were added to bacteria in suspension, but chemiluminescence was also higher when surface-adhered bacteria were opsonized.

When luminol was used as a probe, the PMN chemiluminescence response to opsonized bacteria in suspension was two times higher than the average response elicited by opsonized bacteria adhered to glass ($P < 0.02$). Since luminol-enhanced chemiluminescence reflects an MPO-mediated reaction, opsonized bacteria in suspension may be better activators of the MPO-related oxidative burst than bacteria adhered to a surface.

We confirmed that suspended encapsulated *S. pneumoniae* cells are resistant to phagocytosis in the absence of opsonins (12, 16). Purified *S. pneumoniae* capsular polysaccharide was found to inhibit human PMN phagocytosis of pneumococcus strains (7). However, opsonized *S. pneumoniae* 7F stimulated greater PMN luminol-enhanced chemiluminescent response than *S. pneumoniae* R36, which suggests that *S. pneumoniae* capsular polysaccharide enhances the MPO-dependent response of PMN. There was no difference in luminol-enhanced PMN chemiluminescence between *H. influenzae* type b and non-type b. Therefore, the polyribose phosphate capsule associated with *H. influenzae* type b does not enhance the MPO-dependent chemiluminescent response of PMN.

Lucigenin-enhanced PMN chemiluminescence reflects superoxide (O_2^-) production (11, 14). We found that opsonized bacteria in suspension and adhered to a surface stimulated a nearly equal lucinogen-enhanced chemiluminescent response. It was noted that the PMN lucigenin-enhanced chemiluminescence elicited by unencapsulated *S. pneumoniae* R36A was higher than that by encapsulated *S. pneumoniae* 7F ($P < 0.05$), suggesting that pneumococcal capsular polysaccharide does not contribute to superoxide production by PMN. The response elicited by *H. influenzae* type b was equal to the response elicited by *H. influenzae* non-type b in both the suspension and adherent assays.

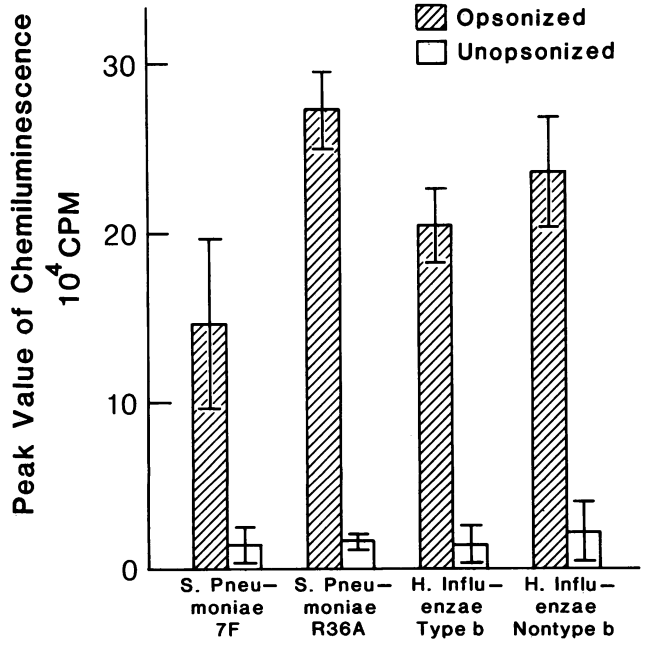

FIG. 3. Peak values of lucigenin-enhanced suspension chemiluminescence (10^4 cpm/10^4 PMN). Opsonized bacteria were pretreated with 10% PHS. Bars indicate SEM.

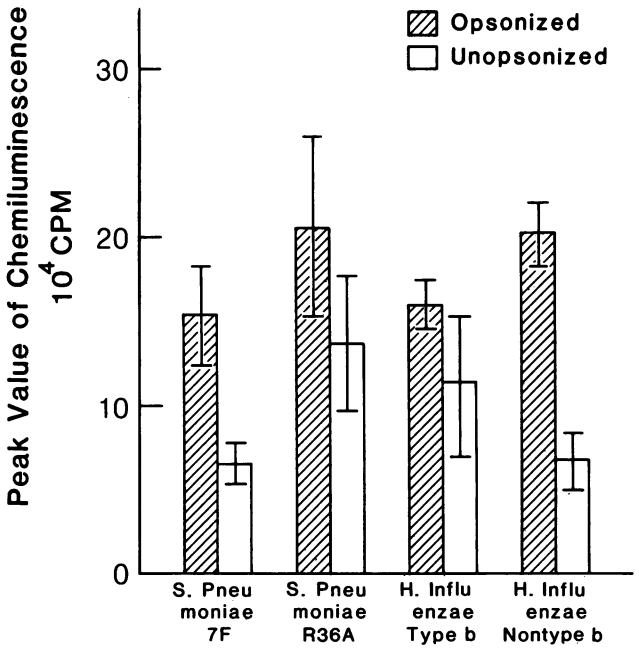

FIG. 4. Peak values of lucigenin-enhanced surface chemiluminescence (10^4 cpm/10^4 PMN). Opsonized bacteria were pretreated with 10% PHS. Bars indicate SEM.

Human PMN respond differently to bacterial species depending on the physical presentation of the bacteria, i.e., adhered to a surface versus in suspension, the association with humoral factors, i.e., opsonized versus unopsonized, and the presence or absence of capsular material. All of these variables are present under in vivo conditions and may influence the pathogenesis of infectious lesions.

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