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Chemiluminescence by Human Alveolar Macrophages: Stimulation with Heat-Killed Bacteria or Phorbol Myristate Acetate

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Chemiluminescence of human alveolar macrophages (AM) was evaluated in vitro. Unstimulated AM generated chemiluminescence that remained constant during incubation. Addition of heat-killed *Staphylococcus aureus* 502A (HKB) or a chemical agent, phorbol myristate acetate, produced high rates of chemiluminescence that were significantly ($P < 0.05$) increased over unstimulated AM. Phorbol myristate acetate- and HKB-stimulated increases in AM chemiluminescence were completely blocked by the enzyme superoxide dismutase. In comparison with unstimulated polymorphonuclear leukocytes, unstimulated AM had significantly ($P < 0.05$) greater levels of chemiluminescence. However, after stimulation by phorbol myristate acetate or HKB, AM showed less chemiluminescence than similarly treated polymorphonuclear leukocytes.

After particle ingestion, metabolic processes are stimulated in phagocytic cells. These biochemical activities include oxygen consumption, glucose oxidation via the hexose monophosphate shunt, and superoxide anion formation (4, 6, 12). In conjunction with these events, emission of light or chemiluminescence occurs in polymorphonuclear leukocytes (PMN) and monocytes (1, 2, 16). This light emission is inhibited by superoxide dismutase (SOD), presumably as a result of rapid degradation of superoxide anions (O_2^-) by SOD (8). Previous studies have demonstrated that phorbol myristate acetate (PMA), a surface-active agent in croton oil, stimulates metabolic responses activated in PMN by particulate ingestion. PMA causes PMN to consume oxygen, oxidize [1-^{14}C]glucose, generate superoxide, and chemiluminesce (5, 17-19). To further characterize the metabolic activities of alveolar macrophages (AM), we investigated chemiluminescence of AM stimulated by particulates or PMA.

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MATERIALS AND METHODS

Preparation of PMN. Venous blood (25 ml) from seven healthy, nonmedicated adult donors was collected in plastic syringes containing 1,000 U of heparin. PMN were obtained by dextran sedimentation (17) with hypotonic lysis of erythrocytes, followed by washing and suspension in Hanks balanced salt solution (HBSS).

Preparation of AM. Four patients having fiberoptic bronchoscopy underwent segmental saline lavage by the method of Finley and co-workers (7). Patients whose AM were studied included a 35-year-old white female smoker with hilar adenopathy, a 53-year-old white male with chronic bronchitis, a 28-year-old white female smoker with a lung nodule, and a 58-year-old white male smoker with carcinoma of the lung. Lavage fluid was poured through a steel mesh and centrifuged at 180 × *g* for 10 min at 0°C. The resulting pellet was resuspended in HBSS. Giemsa-stained smears of the pellet revealed a population of greater than 90% macrophages. When assessed by trypan blue exclusion, the viability of AM was greater than 95%.

Preparation of serum. Serum from type AB, nonmedicated, asymptomatic donors was prepared by a previously described method (17).

Preparation of bacteria. *Staphylococcus aureus* 502A was prepared and heat-killed by a standard technique (17).

Preparation of PMA. PMA (Consolidated Midland Corp., Brewster, N.Y.) was dissolved in dimethyl sulfoxide at a concentration of 2 mg/ml at 4°C. Before use, 5 μl of the PMA-dimethyl sulfoxide solution was diluted with 2 ml of HBSS.

Preparation of SOD. SOD (3,000 U/mg; Sigma Chemical Co., St. Louis, Mo.) was dissolved in HBSS to a final stock solution of 1,000 μg/ml.

Measurement of chemiluminescence. Chemiluminescence was measured at ambient temperature (22°C) in a liquid scintillation counter (Model LS-233, Beckman Instruments, Irvine, Calif.). The coincidence circuit was turned off, leaving only one photomultiplier tube active (22). Counting vials (161698, Beckman Instruments), which had been kept in a dark room for 24 h prior to use, were filled

in the presence of red light illumination. At zero minutes, 10^7 phagocytes were added to the incubation mixtures. The vials were then counted immediately and sequentially every 5 or 8 min. The final incubation mixture contained 10^7 PMN or AM in 2.0 ml of HBSS and 7.5% control pooled serum. Fifty heat-killed bacteria (HKB) per phagocyte, 0.5 μg of PMA per ml, and/or 100 μg of SOD were added to various test vials. A concentration of dimethyl sulfoxide (0.25%) equivalent to that used in the final PMA solution was also added to control vials. Background counts (vials containing 2 ml of HBSS) were subtracted from test samples, and resultant counts were expressed as counts per minute per 10^7 phagocytes. The results for AM from four patients or for PMN from seven donors were averaged and compared by unpaired t-test analysis.

RESULTS

Human AM produced chemiluminescence (Fig. 1). Initially, light emission by AM incubated in HBSS and serum (unstimulated) was 8,700 ± 2,100 cpm/10^7 phagocytes (mean ± 1 standard error of the mean). Light emission by unstimulated AM did not change during incubation. After incubation for 40 min, chemiluminescence of unstimulated AM was 7,000 ± 1,800 cpm. Addition of HKB increased chemiluminescence by AM to 13,900 ± 2,300 cpm after 5 min and to 19,400 ± 2,500 cpm at 40 min. Rates of chemiluminescence by HKB-stimulated AM were significantly ($P < 0.05$) greater than those of unstimulated AM after 15 min of incubation.

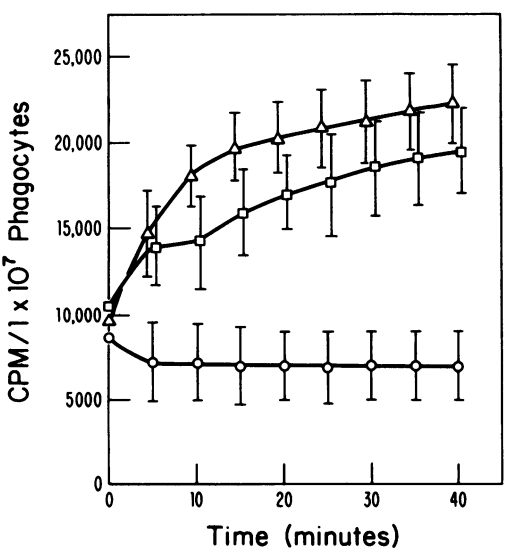

FIG. 1. *Chemiluminescence by human AM. Unstimulated AM* (○), *AM with HKB* (□), *or AM with PMA* (△) *were evaluated. Each point represents the mean ± 1 standard error of the mean for the AM from four patients.*

PMA also stimulated chemiluminescence by AM. After incubation with PMA, AM generated chemiluminescence at levels significantly ($P < 0.05$) increased over unstimulated AM. Chemiluminescence by PMA-treated AM increased continuously during incubation, reaching a level of 22,300 ± 2,300 cpm at 40 min. Chemiluminescence of AM stimulated by PMA was not significantly ($P > 0.1$) different from that of AM stimulated by HKB. When present at concentrations comparable to those in the PMA incubations, dimethyl sulfoxide did not stimulate chemiluminescence by AM. HKB in the absence of PMN or AM did not generate chemiluminescence.

Effect of SOD on chemiluminescence by AM. Chemiluminescence by AM was studied in the presence of 100 μg of SOD per ml (Fig. 2). SOD caused only a small change in light emission by unstimulated AM (Fig. 2a). However, SOD significantly ($P < 0.05$) depressed chemiluminescence generated by AM treated with HKB at 10, 35, and 40 min (Fig. 2b). Similarly, SOD completely inhibited chemiluminescence by PMA-stimulated AM at all times of study (Fig. 2c).

Comparison of chemiluminescence by AM and PMN. Chemiluminescence of AM was compared with that of PMN (Fig. 3). At each time interval, chemiluminescence by unstimulated AM was significantly ($P < 0.05$) greater than by unstimulated PMN (Fig. 3a). Addition of HKB caused only a small increase in chemiluminescence by AM compared with the marked stimulation of chemiluminescence in HKB-treated PMN (Fig. 3b). Chemiluminescence by HKB-stimulated PMN was significantly ($P < 0.05$) greater than for HKB-treated AM or unstimulated PMN. Although PMA increased chemiluminescence by PMN or AM, the kinetics of chemiluminescence production indicated a difference in the activation and response of these two cells (Fig. 3c). PMA caused a rapid initial burst of chemiluminescence in PMN, with subsequent fall-off to a lower sustained level. In AM stimulated by PMA, there was no initial burst, only a steady increase that plateaued during later incubation times.

DISCUSSION

In the present investigation, we found that human AM chemiluminesce. Light emission was observed for unstimulated AM or AM stimulated by addition of particulates or PMA. We also demonstrated that chemiluminescence of stimulated AM was inhibited by addition of SOD.

The observation that human AM chemilumi-

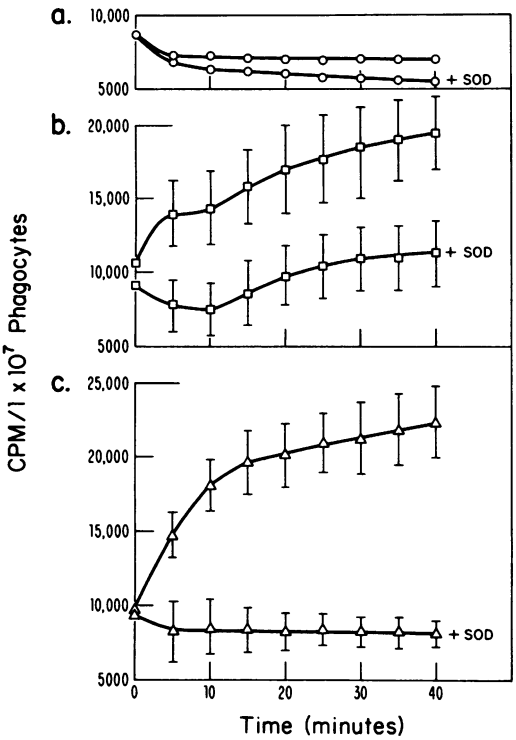

FIG. 2. *Effect of SOD on chemiluminescence by AM. Unstimulated AM (a), AM with HKB (b), or AM with PMA (c) were studied alone and in the presence of 100 μg of SOD per ml. Each point represents the mean ± 1 standard error of the mean for the AM from four patients.*

nesce has important implications. Previous work by other investigators has suggested a central role for a number of high-energy oxygen compounds in the production of chemiluminescence by PMN; these compounds include O_2^- (1, 5, 23), singlet oxygen (2, 9), hydroxyl radicals (23), and hypochlorite anions (20). Since human AM chemiluminesce, it appears they are capable of generating similar high-energy compounds. Furthermore, it is known that SOD can specifically degrade O_2^- and inhibit O_2^--associated chemiluminescence (8, 9, 23). In view of SOD's specificity and marked inhibition of AM chemiluminescence, it appears that increased chemiluminescence by stimulated AM is due primarily to O_2^- or O_2^--derived compounds. Thus, this study suggests that superoxide formation is occurring in AM as well as in PMN or monocytes (6, 21). The present results substantiate previous findings that demonstrated O_2^- production by AM in a cytochrome *c* oxidation assay (6).

Another interesting aspect of this study is the effect of PMA stimulation on chemiluminescence by AM. PMA proved a potent stimulus to AM chemiluminescence. These experiments further demonstrate the usefulness of PMA by showing that this agent can activate metabolic activities of human AM as well as those of PMN (5, 17–19) or monocytes (11, 21).

Insight into the nature of the metabolic activities of AM and PMN can be gained from a comparison of their chemiluminescence. This investigation showed that, although unstimulated AM have greater chemiluminescence than unstimulated PMN, AM show less response to stimulation than PMN. Whether these differences are a result of alteration of AM by the lavage procedure or the patient's condition cannot be determined. However, these findings parallel results of other studies of oxidative metabolism; compared with PMN, AM have higher unstimulated levels of oxygen consumption and [1-^{14}C]glucose oxidation but show less stimulation after phagocytosis (12). In addition, there is a difference in the response of PMN and AM to PMA. In PMN stimulated by PMA, there is a marked initial burst in chemiluminescence, followed by a fall-off to a

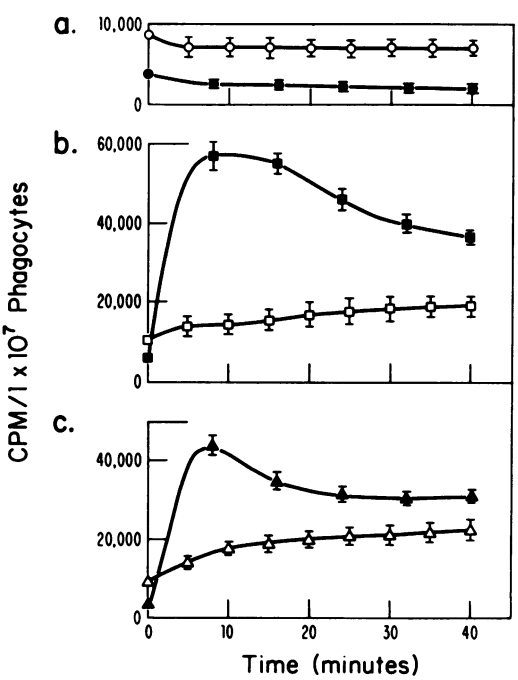

FIG. 3. *Comparison of chemiluminescence by AM and PMN. (a) Unstimulated AM (○) or PMN (●), (b) AM (□) or PMN (■) with HKB, or (c) AM (△) or PMN (△) with 0.5 μg of PMA per ml were compared. Each point represents the mean ± 1 standard error of the mean for PMN from seven donors or AM from four patients.*

lower sustained level. In PMA-treated AM, there is no initial burst, only a gradual increase.

The exact significance of these differences in chemiluminescence between PMN and AM is unknown. In PMN, many chemiluminescence-producing compounds may be involved in killing of bacteria either directly (3, 10, 14, 15, 20) or through H_2O_2 formation (13). Thus, in PMN, chemiluminescence appears to measure production of potentially bactericidal compounds. Although the bactericidal mechanisms of AM are not well understood, the chemiluminescence of AM suggests that light-generating compounds are available and may contribute to the bactericidal capabilities of these cells.

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LITERATURE CITED

1. Allen, R. C., R. J. Stjernham, and R. H. Steele. 1972. Evidence for the generation of an electronic excitation state(s) in human polymorphonuclear leukocytes and its participation in bactericidal activity. Biochem. Biophys. Res. Commun. 47:679–684.
2. Allen, R. C., S. J. Yevich, R. W. Orth, and R. H. Steele. 1974. The superoxide anion and singlet molecular oxygen: their role in the microbicidal activity of the polymorphonuclear leukocyte. Biochem. Biophys. Res. Commun. 60:909–917.
3. Babior, B. M., J. T. Curnutte, and R. S. Kipnes. 1975. Biological defense mechanisms. Evidence for the participation of superoxides in bacterial killing by xanthine oxidase. J. Lab. Clin. Med. 85:235–244.
4. Babior, B. M., R. S. Kipnes, and J. T. Curnutte. 1973. Biological defense mechanism; the production by leukocytes of superoxide, a potent bactericidal agent. J. Clin. Invest. 52:741–744.
5. DeChatelet, L. R., P. S. Shirley, and R. B. Johnston, Jr. 1976. Effect of phorbol myristate acetate on the oxidative metabolism of human polymorphonuclear leukocytes. Blood 47:545–554.
6. Drath, D. B., and M. Karnovsky. 1975. Superoxide production by phagocytic leukocytes. J. Exp. Med. 141:257–262.
7. Finley, T. N., E. W. Swenson, W. S. Curran, G. L. Huber, and A. J. Ladman. 1967. Bronchopulmonary lavage in normal subjects and patients with obstructive lung disease. Ann. Intern. Med. 66:651–658.
8. Fridovich, I. 1975. Superoxide dismutases. Annu. Rev. Biochem. 44:147–159.
9. Goda, K., J. Chu, T. Kimura, and A. P. Schaap. 1973. Cytochrome C enhancement of singlet molecular oxygen production by the NADPH-dependent adrenodoxin reductase-adrenodrenodoxin system: the role of singlet oxygen in damaging mitochrondrial membranes. Biochem. Biophys. Res. Commun. 52:1300–1306.
10. Johnston, R. B., Jr., B. B. Keele, H. P. Misra, J. E. Lehmeyer, L. S. Webb, S. Lawrence, R. L. Baehner, and K. V. Rajagopalan. 1975. The role of superoxide anion generation in phagocytic bactericidal activity: studies with normal and chronic granulomatous disease leukocytes. J. Clin. Invest. 55:1357–1372.
11. Johnston, R. B., Jr., J. E. Lehmeyer, and L. A. Guthrie. 1976. Generation of superoxide anion and chemiluminescence by human monocytes during phagocytosis and on contact with surface-bound immunoglobulin G. J. Exp. Med. 143:1551–1556.
12. Karnovsky, M. L. 1962. Metabolic basis of phagocytic activity. Physiol. Rev. 24:143–168.
13. Klebanoff, S. J. 1974. Role of the superoxide anion in the myeloperoxidase-mediated antimicrobial system. J. Biol. Chem. 249:3724–3728.
14. Klebanoff, S. J. 1975. Antimicrobial mechanisms in neutrophilic leukocytes. Semin. Hematol. 12:117–142.
15. Krinsky, N. I. 1974. Singlet excited oxygen as a mediator of the antibacterial action of leukocytes. Science 186:363–365.
16. Nelson, R. D., E. L. Mills, R. L. Simmons, and P. G. Quie. 1976. Chemiluminescence response of phagocytizing human monocytes. Infect. Immun. 14:129–134.
17. Repine, J. E., J. G. White, C. C. Clawson, and B. M. Holmes. 1974. The influence of phorbol myristate acetate on oxygen consumption by polymorphonuclear leukocytes. J. Lab. Clin. Med. 83:911–920.
18. Repine, J. E., J. G. White, C. C. Clawson, and B. M. Holmes. 1974. Effects of phorbol myristate acetate on the metabolism and ultrastructure of neutrophils in chronic granulomatous disease. J. Clin. Invest 54:83–90.
19. Repine, J. E., J. G. White, C. C. Clawson, and B. M. Holmes. 1975. The influence of phorbol myristate acetate on the metabolism of neutrophils from carriers of sex-linked chronic granulomatous disease. J. Lab. Clin. Med. 85:82–86.
20. Rosen, H., and S. J. Klebanoff. 1976. Chemiluminescence and superoxide production by myeloperoxidase-deficient leukocytes. J. Clin. Invest. 58:50–60.
21. Sagone, A. L., Jr., G. W. King, and E. N. Metz. 1976. A comparison of the metabolic response to phagocytosis in human granulocytes and monocytes. J. Clin. Invest. 57:1352–1358.
22. Stanley, D. E., and S. G. Williams. 1969. Use of the scintillation spectrometer for determining adenosine triphosphate by the luciferase enzyme. Anal. Biochem. 29:381–392.
23. Webb, L. W., B. B. Keele, Jr., and R. B. Johnston, Jr. 1974. Inhibition of phagocytosis-associated chemiluminescence by superoxide dismutase. Infect. Immun. 9:1051–1056.