Brief Definitive Report

GENERATION OF SUPEROXIDE ANION AND CHEMILUMINESCENCE BY HUMAN MONOCYTES DURING PHAGOCYTOSIS AND ON CONTACT WITH SURFACE-BOUND IMMUNOGLOBULIN G*

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Polymorphonuclear neutrophils and monocytes emigrate from the bloodstream to areas of inflammation and play an essential role there in a variety of physiological and pathological events, including defense against infection. Although the biochemical basis for these cells' microbicidal activity is not completely understood, studies primarily with neutrophils indicate that the killing of most organisms depends upon phagocytosis-associated oxidative metabolism. During phagocytosis neutrophils remove oxygen from the surrounding medium and convert it, probably first, to superoxide anion $(O_2^- \cdot)$ (1, 2) and then to hydrogen peroxide (H_2O_2) (3). H_2O_2 and $O_2^- \cdot$ may interact to form the potent oxidant, hydroxyl radical $(\cdot OH)$ (2). Oxidation of glucose through the hexose monophosphate (HMP) shunt is stimulated, perhaps as a result of increased H_2O_2 generation (4). The spontaneous dismutation of $O_2^- \cdot$ is believed to result in singlet oxygen formation (reviewed in 2), and the occurrence of this reaction in neutrophils appears to be the most likely explanation for the luminescence that occurs with phagocytosis (2, 5).

Like neutrophils, monocytes undergo increased HMP shunt activation (6–8), oxygen consumption (7, 8), and H_2O_2 generation (7) during ingestion. Macrophages from mice and guinea pigs generate $O_2^- \cdot$ (9). We report here that human monocytes elaborate $O_2^- \cdot$ and generate chemiluminescence during phagocytosis, on stimulation by a surface-active chemical agent, and on contact with fixed aggregated IgG.

Materials and Methods

Preparation of Cells. Human neutrophils were separated from other leukocytes in 96–99% purity by centrifugation of defibrinated or heparinized venous blood through a Ficoll-Hypaque mixture (2) at 310 g for 35 min at room temperature. Erythrocytes were removed by dextran sedimentation and hypotonic lysis (2), and neutrophils were washed and suspended in Krebs-Ringer phosphate buffer, pH 7.35, containing 0.2% glucose and 0.2% bovine serum albumin. Monocyte-lymphocyte layers from the same preparations were diluted with 4 vol of buffer and centrifuged at 370 g, 4°C for 10 min. Erythrocytes were removed by hypotonic lysis, and the

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leukocytes were washed twice and suspended in buffer. The percentages of monocytes were determined by differential counting of 600 cells on smears stained with Wright's stain. These preparations, termed "monocytes" in this paper, contained 20–43% monocytes (mean, 30%), <1% granulocytes, and the remainder lymphocytes. Results with phagocytes from defibrinated or heparinized blood did not differ.

To obtain lymphocyte preparations, the pelleted mononuclear leukocytes (above) were resuspended to the original blood volume in buffer containing 5% heat-inactivated fetal calf serum (Grand Island Biological Co., Grand Island, N. Y.), and this suspension was divided into 25-ml fractions, each of which was incubated in a 500-ml flask at 37°C, 5% CO_2 for 60 min to permit monocyte adherence. This incubation was repeated until monocytes comprised <5% (usually <3%) of leukocytes. The purified lymphocytes were washed and resuspended in buffer.

Preparation of Stimulating Agents. Zymosan particles were opsonized by incubation with human serum (2). Phorbol myristate acetate (PMA, Consolidated Midland Corp., Brewster, N. Y.) was dissolved in dimethylsulfoxide (grade I, Sigma Chemical Co., St. Louis, Mo.) and suspended in buffer to a concentration of 1 mg/ml. Dimethylsulfoxide alone had no detectable effect on monocytes or neutrophils. Human IgG was aggregated by heat and fixed to micropore filters (10). The IgG is bound tightly to the filters in this system (10).

Assays. $O_2^-\cdot$ was quantitated by its ability to reduce cytochrome c (1, 10). 2.5×10^6 phagocytes or lymphocytes were incubated with 0.08 mM ferricytochrome c and with either opsonized zymosan or 5 μg PMA at 37°C for 10 min (2), or with IgG-coated filters for 5–120 min (10). Supernatant fluids were assayed spectrophotometrically (2, 10), and results were converted to nanomoles of cytochrome c reduced using $\Delta E_{550\ nm} = 2.1 \times 10^4\ M^{-1}\ cm^{-1}$.

Chemiluminescence was measured in a scintillation spectrometer (2, 10). When the stimulatory agent was opsonized zymosan or PMA, 10^7 phagocytes or lymphocytes were present in a reaction vol of 4 ml (2); 5×10^6 cells in duplicate reaction vol of 1.5 ml were used with IgG-coated filters (10).

Results

Phagocytosis of opsonized zymosan, contact with the surface-active agent PMA, or contact with micropore filters coated with aggregated human IgG stimulated monocytes from human peripheral blood to reduce ferricytochrome c (Table I). That this reduction was due to $O_2^-\cdot$ is indicated by its inhibition with superoxide dismutase. The release of $O_2^-\cdot$ from monocytes was about two-thirds that from neutrophils of the same donor during phagocytosis and on stimulation by PMA (63 and 65%, respectively; $P < 0.005$ for both, paired t test). $O_2^-\cdot$ release from monocytes more nearly approximated that of neutrophils when the IgG-coated filter was the stimulating agent (Table I). Nevertheless, in four paired kinetic experiments, monocytes elaborated significantly less $O_2^-\cdot$ than neutrophils (mean 81%, range 62–95%, at six intervals from 5 to 120 min; $P < 0.005$, analysis of variance).

Five cell preparations containing 97–99% lymphocytes and 1–3% monocytes did not release significant amounts of $O_2^-\cdot$ when incubated with opsonized zymosan or PMA (0.52 and 0.45 nmol/min per 2.5×10^6 lymphocytes, respectively) or when allowed to settle on IgG-coated filters (0.05 nmol/min). Lymphocytes added to neutrophil preparations at a lymphocyte:neutrophil ratio of 2:1 or 3:1, simulating the ratio of lymphocytes to phagocytic cells in monocyte preparations, had no significant effect on $O_2^-\cdot$ release from neutrophils stimulated by any of the three agents.

Monocytes released minimal amounts of $O_2^-\cdot$ on contact with polystyrene Petri dishes (Table I) or micropore filters without attached IgG (0.32 nmol/min, mean of four experiments). In four experiments with IgG-coated filters monocytes were preincubated for 15 min with 5 μg/ml cytochalasin B (10), a fungal

TABLE I

Release of Superoxide Anion ($O_2^-\cdot$) by Human Neutrophils and Monocytes

Stimulating agent	SOD*	Ferricytochrome c reduction‡	
		Neutrophils	Monocytes
		(nmol/min/2.5 × 10^6 phagocytes)	
None	−	0.66 ± 0.32 (6)	0.73 ± 0.36
Opsonized zymosan	−	7.98 ± 0.99 (8)	5.01 ± 1.86
Opsonized zymosan	+	0.34 ± 0.23 (3)	0.54 ± 0.31
PMA	−	9.03 ± 1.11 (7)	5.83 ± 1.64
PMA	+	0.09 ± 0.15 (3)	0.51 ± 0.26
Polystyrene dish	−	0.11 ± 0.07 (4)	0.18 ± 0.08
Filter + aggregated IgG	−	1.35 ± 0.24 (12)	1.14 ± 0.24
Filter + aggregated IgG	+	0.01 ± 0.02 (6)	0.17 ± 0.11

* The presence of 33 µg/ml purified superoxide dismutase (SOD) in the reaction mixture is denoted by +, its absence by −.

‡ $O_2^-\cdot$ was quantitated by its capacity to reduce ferricytochrome c. The inhibition of this reduction by SOD is shown; heat denaturation of SOD removed at least 90% of this inhibitory effect. Values are expressed as mean ± SD of averages of duplicate or triplicate determinations using 2.5 × 10^6 phagocytes in each. The number of paired experiments is given in parentheses in the neutrophil column. Incubation time was 10 min when cells were suspended in tubes with zymosan, PMA, or buffer ("none") and 30 min when cells were allowed to settle onto polystyrene Petri dishes or onto filters coated with aggregated IgG.

metabolite known to interfere with microfilament function. $O_2^-\cdot$ release was reduced by a mean of 53%, presumably due to inhibition of pseudopod formation, spreading, and surface-cell contact (11).

In seven paired experiments monocytes generated chemiluminescence during phagocytosis of opsonized zymosan or on contact with PMA (Fig. 1). The peak levels of luminescence achieved with either stimulus were half to two-thirds those of neutrophils, much like the relationship noted with $O_2^-\cdot$. Lymphocytes did not generate luminescence on exposure to either stimulus (Fig. 1), and the addition of purified lymphocytes to neutrophil preparations in a ratio of 2:1 had no effect on chemiluminescence generation during stimulation by either agent.

Superoxide dismutase has been shown to reduce phagocytosis-associated luminescence by neutrophils by approximately 75%, perhaps through inhibition of singlet oxygen formation during the spontaneous dismutation of $O_2^-\cdot$ (2). In four experiments, the presence of 50 µg/ml superoxide dismutase reduced peak chemiluminescence by monocytes stimulated with opsonized zymosan and PMA by 78 and 79%, respectively, indicating an involvement of $O_2^-\cdot$ in the luminescence generated by monocytes, as well as by neutrophils.

Contact of phagocytic cells with fixed aggregated IgG also induced chemiluminescence (Fig. 2). In contrast to results with $O_2^-\cdot$ release (Table I), and for reasons which are presently obscure, this nonphagocytosable surface was much less stimulatory for monocytes than neutrophils ($P < 0.001$, paired t test for peak values). Lymphocyte preparations did not generate significant chemiluminescence on exposure to IgG on filters. Superoxide dismutase reduced chemiluminescence by both monocytes and neutrophils in this system by approximately 79%.

Discussion

Although the means by which mononuclear phagocytes kill ingested microorganisms has not been well defined, circulating monocytes (7) and alveolar macrophages (12) possess the ability to consume oxygen and convert it to H_2O_2, a process clearly associated with the bactericidal capacity of neutrophils. Recent
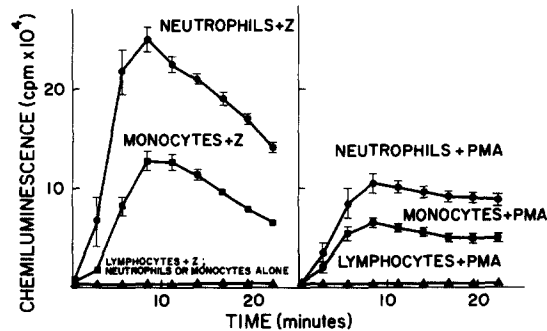
Fig. 1. Generation of chemiluminescence by 10^7 neutrophils, monocytes, or lymphocytes in the presence of opsonized zymosan (Z) or PMA. Mean values are plotted for seven experiments with either stimulus; the bars indicate the SEM. Monocytes were significantly less active than neutrophils in both assays ($P < 0.001$ for zymosan, $P < 0.02$ for PMA, paired t test using peak values).
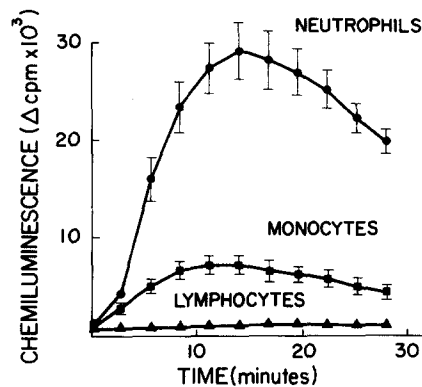

Fig. 2. Generation of chemiluminescence by 5×10^6 neutrophils, monocytes, or lymphocytes in contact with aggregated IgG bound to micropore filters. Means of averaged duplicate values ± the SEM are plotted for seven experiments. A value of 2,000 cpm, representing the mean background for empty vials, has been subtracted. In the absence of IgG-coated filters, values for neutrophils, monocytes, or lymphocytes were not different from those shown for lymphocytes in contact with the filter-IgG.

reports indicate that monocytes, like neutrophils, release $O_2^-\cdot$ (13) and generate chemiluminescence (14) during phagocytosis; and mouse and guinea pig macrophages can release $O_2^-\cdot$ (9). Our results indicate that surface perturbation, as well as phagocytosis, will induce monocytes to elaborate $O_2^-\cdot$ and generate chemiluminescence. Previous comparisons of the intensity of the phagocytosis-associated burst of oxygen metabolism in monocytes and neutrophils have yielded conflicting results (7, 8). We found monocytes to be significantly less active than neutrophils from the same individual in elaborating $O_2^-\cdot$ and generating chemiluminescence with any of the three stimuli used.

Mononuclear phagocytes accumulate in chronic inflammatory lesions, including those associated with infections due to intracellular parasites, certain types of nephritis, rheumatoid arthritis, rejection of allografts, and rejection of tumors to which the host has been immunized (reviewed in 15). Evidence exists that

mononuclear phagocytes play an essential role in the tissue damage or protective cytotoxicity of these conditions (15). In such conditions, the monocyte-macrophage would be expected to bind to fixed immune complexes or to antibody-coated cells through its plasma membrane receptors for C3b or the Fc portion of immunoglobulin, or both. The results reported here suggest that mononuclear phagocytes accumulating at sites of inflammation are capable of elaborating toxic oxygen metabolites into the surrounding tissues. These oxygen by-products could damage tissue through oxidative decomposition of membrane-associated fatty acids, reaction with nucleic acid bases, or destruction of thiol groups (16). These same mechanisms might also contribute to the capacity of mononuclear phagocytes to participate in the rejection of allografts and the elimination of neoplastic cells.

Summary

Extent of $O_2^-\cdot$ release and chemiluminescence, attributed to singlet oxygen, has been compared in human monocytes and neutrophils during phagocytosis, stimulation by the surface-active agent phorbol myristate acetate, or contact with aggregated IgG in a model of immune complex disease. Monocytes generated $O_2^-\cdot$ and chemiluminescence with each of the three stimuli, although values were significantly less than those of neutrophils from the same individuals. Lymphocytes had no significant activity in either assay with any stimulus. Oxygen metabolites released from mononuclear phagocytes are highly reactive and could play a part in both the beneficial and detrimental aspects of chronic inflammation.

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