Elaboration of Toxic Oxygen By-Products by Neutrophils in a Model of Immune Complex Disease

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ABSTRACT Contact between human neutrophils and aggregated immunoglobulin G bound to micropore filters has been studied as a model of the pathogenesis of tissue damage in immune complex disease. Contact with this surface, as well as with plain filters and polystyrene petri dishes, induced neutrophils to elaborate superoxide anion and hydrogen peroxide and to generate chemiluminescence, which has been attributed to singlet oxygen. Pretreatment of the cells with cytochalasin B decreased these activities but increased release of lysosomal β-glucuronidase, suggesting that degranulation and the burst of oxygen metabolism that characterizes phagocytes are independently regulated functions. Toxic oxygen metabolites released from neutrophils are highly reactive and could mediate tissue injury at sites of inflammation.

INTRODUCTION

A common feature of acute inflammation is the local accumulation of polymorphonuclear neutrophils (PMNs).[1] When the inflammation is infectious in origin, PMNs may play an essential role in host defense. The possibility has been raised, however, that these cells also cause a major part of the tissue injury that occurs with inflammation; and dependence upon PMNs for development of the inflammatory manifestations of serum sickness arteritis (1), Arthus reactions (2, 3) and antibody-mediated nephritis (4) has been described. The occurrence of PMN lysosomal proteases and cationic proteins in urine of animals with nephrotoxic nephritis suggests that PMN-mediated tissue injury may result from lysosomal degranulation (5), at least in this disease. However, electron micrographs of the PMNs that line the site of renal injury have not shown granular depletion (4), and the possibility exists that other PMN factors may play a role in inflammatory tissue damage.

When inflammation has an immunologic origin, antigen, antibody, and complement, as well as PMNs, can be demonstrated at the inflammatory site. An in vitro model for studying immunologic tissue injury utilizes immune complexes or aggregated immunoglobulin fixed to the surface of a micropore filter (6, 7) or tissue such as cartilage (8) or collagen (9). In this model PMNs adhere to the nonphagocytosable surface and discharge their lysosomal contents at or near the site of attachment.

Using this system, we have found that activation of human PMNs by contact with polystyrene, micropore filters, or aggregated immunoglobulin bound to filters results in vigorous generation of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and chemiluminescence.

METHODS

Neutrophils. PMNs were separated from heparinized venous blood, freed of erythrocytes and platelets, and washed with Krebs-Ringer phosphate buffer with 2 mg/ml each dextrose and albumin (KRP-DA), as previously described (10). In some experiments cells were preincubated with 5 μg/ml of the fungal metabolite cytochalasin B (Aldrich Chemical Co., Inc., Milwaukee, Wis.) for 15 min at 37°C. The cytochalasin B was dissolved in dimethylsulfoxide (DMSO; grade I, Sigma Chemical Co., St. Louis, Mo.) and diluted with 2 vol KRP-DA buffer to a concentration of 1 mg/ml. Control cells were preincubated with an equal concentration of DMSO; however, results in any of the three assays described below did not differ from those obtained when cells were preincubated in buffer without DMSO.

Filters. Micropore filters composed of mixed esters of cellulose, 25 mm diameter, 5 μm pore size (Millipore Corp., Bedford, Mass., catalog no. SMWP 02500), were incubated for 60 min at room temperature with phosphate-buffered saline, pH 7.4 (PBS), or with heat-aggregated (63°C, 15 min) human immunoglobulin G (IgG) (11), 2–3 mg/ml in

Received for publication 14 August 1975 and in revised form 11 December 1975.

[1] *Abbreviations used in this paper:* DMSO, dimethylsulfoxide; HMP, hexose monophosphate; IgG, immunoglobulin G; KRP-DA, Krebs-Ringer phosphate buffer with dextrose and albumin; LDH, lactate dehydrogenase; O_2^-, superoxide anion; PBS, phosphate-buffered saline; PMNs, polymorphonuclear neutrophils.

TABLE I

Release of O_2^-, β-Glucuronidase, and LDH by PMNs in Contact with Nonphagocytosable Surfaces

Nonphagocytosable surface	Cytochalasin B*	Ferricytochrome *c* reduction‡		Enzyme activity released§	
		−SOD	+SOD	β-Glucuronidase	LDH
		nmol/min per 5 × 10^6 phagocytes		%	
Polystyrene	−	0.336±0.259 (9)	0.099±0.122 (3)	3.0±2.4 (12)	2.9±1.8 (9)
Polystyrene	+	0.139±0.116 (12)	0.075±0.113 (3)	5.0±3.9 (17)	3.2±1.5 (10)
Filter	−	0.520±0.266 (11)	0.041±0.060 (3)	2.6±1.7 (16)	3.2±1.4 (11)
Filter	+	0.181±0.185 (22)	0.048±0.082 (3)	5.1±3.0 (27)	3.6±1.5 (14)
Filter + aggregated IgG	−	0.775±0.171 (7)	0.138±0.185 (4)	5.2±2.7 (14)	3.4±1.5 (9)
Filter + aggregated IgG	+	0.569±0.201 (19)	0.062±0.093 (15)	10.0±3.3 (27)	3.8±1.3 (11)

* Granulocytes were preincubated with cytochalasin B, 5 μg/ml, (denoted by +) or buffer with DMSO (−), as described in Methods.

‡ O_2^- was quantitated by its capacity to reduce ferricytochrome *c*. The inhibition of this reduction by 15 μg/ml purified superoxide dismutase (SOD) is shown; heat denaturation of the enzyme removed 90–100% of this inhibitory effect. Values are expressed as mean±SD of averages of duplicate or triplicate determinations; the number of experiments is given in parentheses.

§ Enzyme activity released by PMNs on surface contact is expressed as percent of total enzyme activity released from an equal number of cells by sonication. Means±SD of averages of duplicate or triplicate determination are shown; the number of experiments is given in parentheses.

PBS. After incubation all filters were washed five times by gentle swirling in 10–12 ml of PBS and placed in 35 × 10-mm polystyrene petri dishes (Falcon Plastics, Oxnard, Calif., catalog no. 1008).

The IgG was bound tightly to the filters: (*a*) When ^{125}I-IgG was used, 5% or less of the radioactivity on the filter appeared in the final wash or in 1.5 ml buffer incubated with the filter for 60 min; (*b*) neither of these fluids stimulated O_2^- or chemiluminescence generation when incubated with PMNs for 60 min; and (*c*) preincubating the IgG-coated filters with 1.5 ml buffer for 60 min resulted in no detectable loss in their capacity to stimulate O_2^- release from PMNs.

Assays. The reaction was begun by adding PMNs in 1.5 ml KRP-DA buffer to the petri dishes. After incubation at 37°C without shaking for 60 min, the supernatant liquid was transferred to a chilled tube which was promptly centrifuged at 180 *g* for 10 min at 4°C.

In experiments studying O_2^- formation, 0.08 mM ferricytochrome *c* (type III, Sigma Chemical Co.) was present with 5 × 10^6 PMNs during incubation, and supernatant fluids were assayed for reduced cytochrome *c* by measurement of absorbance at 550 nm (10, 12). Dishes containing only buffer and ferricytochrome *c*, incubated as above, served as blanks. Results with duplicate or triplicate mixtures were averaged and converted to nanomoles of cytochrome *c* reduced by using $\Delta E_{550\ nm} = 2.1 \times 10^4\ M^{-1}\ cm^{-1}$ (13). Superoxide dismutase, purified by the method of McCord and Fridovich (14), was present in some mixtures at a concentration of 15 μg/ml. Enzyme denatured by autoclaving for 10 min at 124°C served as a control.

Supernates were also assayed for activity of the granular enzyme β-glucuronidase (EC 3.2.1.31) (15) and the cytoplasmic enzyme lactate dehydrogenase (LDH, EC 1.1.1.27) (16). Averaged results from duplicate or triplicate reaction mixtures were expressed as percent of the total activity of these enzymes released from an equal number of PMNs by sonication for 20 s with a Branson W185 sonifier with microtip, power setting 55–60 W (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.).

Release of H_2O_2 by 2.5 × 10^6 PMNs was measured in supernates from separate petri dishes by quantitating the decrease in fluorescence intensity of scopoletin due to its peroxidase-mediated oxidation by H_2O_2 (17, 18). Sodium azide, 1 mM, was present in these reaction mixtures to inhibit the decay of generated H_2O_2 (19). The assay system consisted of 0.1 ml of 50 μM scopoletin (Sigma Chemical Co.) in PBS, 0.1 ml of supernate or PBS, and 0.2 ml of either PBS or 100 U catalase (Sigma Chemical Co.) in PBS. After incubation for 30 min at room temperature, 0.1 ml horseradish peroxidase (type II, Sigma Chemical Co.), 1 mg/ml in PBS, was added, and fluorescence intensity of the scopoletin was quantitated in a fluorescence spectrophotometer (17, 18). In verification of Andreae (17), we found that 1 mol of H_2O_2 oxidized 1 mol of scopoletin, and this relationship was used to calculate H_2O_2 concentration in the samples. Preincubation of supernates with an excess of catalase (100 U), which removes H_2O_2, eliminated virtually all fluorescence reduction.

Chemiluminescence was measured in a scintillation spectrometer, as previously described (10), except that particles for ingestion were replaced by plain or IgG-coated filters, and 5 × 10^6 PMNs were used in a reaction volume of 1.5 ml. Vials were counted for 1-min intervals without mixing in between.

RESULTS

As summarized in Table I, contact with the surface of a polystyrene petri dish, a micropore filter, or a micropore filter coated with IgG stimulated PMNs to reduce ferricytochrome *c*. That this reduction was due to O_2^- was indicated by its inhibition in the presence of superoxide dismutase (14). The stimulatory effect of the IgG-coated filters was significantly greater than that of either of the other two surfaces, whether or not the PMNs had been pretreated with cytochalasin B ($P < 0.001$, analysis of variance [20]). Cytochalasin B treatment significantly reduced O_2^- generation with each of the three surfaces ($P < 0.001$, analysis of variance).

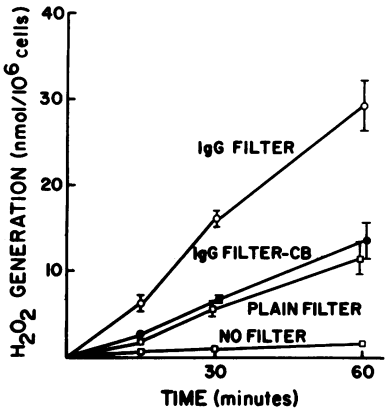

FIGURE 1 Generation of H_2O_2 by PMNs contacting nonphagocytosable surfaces, plotted as a function of the length of incubation. 2.5×10^6 PMNs were used, and triplicate reaction mixtures were studied. The means of averaged values are plotted for six experiments; the bars above and below each point represent the SEM. "CB" indicates that the cells were treated with cytochalasin B. Results with IgG-coated filters were significantly greater than those achieved with plain filters or no filters, whether rate ($P < 0.001$) or peak values ($P < 0.001$) were analyzed (Duncan's multiple-range test [20]). With IgG-coated filters, cytochalasin B treatment reduced peak H_2O_2 generation by 54% ($P < 0.001$).

The O_2^- production by PMNs incubated in polystyrene dishes was primarily the result of stimulation by contact with the dish rather than basal, "resting" activity: The same number of untreated cells suspended in polypropylene tubes at 37°C for 60 min elaborated minimal amounts of O_2^- (0.149±0.107 nmol/min, mean±SD, five experiments), and siliconizing the dish decreased O_2^- release from untreated cells by 50% and from cytochalasin B-treated cells by almost 100%. The release of O_2^- from cytochalasin B-treated cells in polypropylene tubes (0.111 ±0.105 nmol/min, mean±SD) was not significantly different than that of untreated cells (above) in five paired experiments ($P > 0.5$, Student's t test [20]). The presence of a plain micropore filter in the petri dish increased O_2^- generation, presumably because contact area was increased.

Cells in contact with any surface released the granular enzyme β-glucuronidase (Table I). The extent of release was increased significantly by treatment with cytochalasin B when the surface studied was the filter or the IgG-coated filter ($P < 0.001$, analysis of variance) but not when the surface was the polystyrene dish ($P = 0.1$). Release of LDH from the cytoplasm was consistently low with any of the three surfaces, indicating that there was no significant loss of cell viability. The extent of LDH released did not vary significantly with the presence or absence of cytochalasin B ($P > 0.5$, analysis of variance). β-Glucuronidase release from cells suspended in polypropylene tubes at 37°C for 60 min was minimal with or without pretreatment with cytochalasin B (3.4± 2.1% and 2.7±1.3%, mean±SD, respectively), as was LDH release (2.7±1.4% and 2.6±1.5%, respectively, six paired experiments with both enzymes).

H_2O_2 was also generated by surface-activated PMNs, as depicted in Fig. 1. As with O_2^- release, micropore filters with attached aggregated IgG induced more H_2O_2 release than did plain filters or polystyrene petri dishes alone. The kinetics of H_2O_2 release with IgG-coated filters were similar to those for O_2^- generation (data not shown).

Neutrophils phagocytizing particles emit luminescence that can be quantitated in a liquid scintillation spectrometer (10, 21). Contact with nonphagocytosable surfaces also induced chemiluminescence (Fig. 2). Superoxide dismutase, 3–30 μg/ml, reduced peak chemiluminescence in this system to a mean of 28% of control (four experiments), presumably by preventing the singlet oxygen formation that occurs with the spontaneous dismutation of O_2^- (reviewed in reference 10). Heat-denatured enzyme had no inhibitory activity. Inhibition to a similar extent by superoxide dismutase has been shown for phagocytosis-associated chemiluminescence (10).

Because incubation of PMNs in polystyrene dishes resulted in more O_2^- generation than incubation in polypropylene tubes ($P = 0.03$, sign test [20]), attempts were made to determine if dish surfaces contained endotoxin or other stimulatory material, as follows: (*a*) KRP-DA buffer was incubated at 37°C for 60 min with a random sampling of dishes and polypropylene tubes. The buffer from each dish and tube was then used to

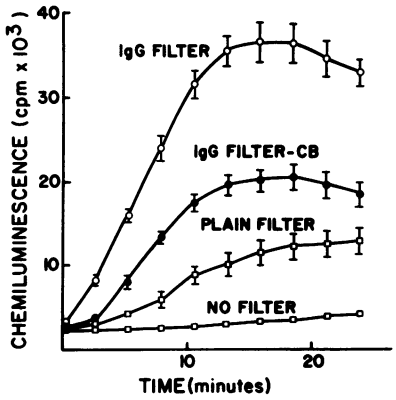

FIGURE 2 Generation of chemiluminescence by PMNs contacting nonphagocytosable surfaces, plotted as a function of the length of incubation. 5×10^6 PMNs were used, and duplicate reaction mixtures were studied. The means of averaged values ±SEM are plotted for seven experiments. Results with IgG-coated filters were significantly greater than those achieved with plain filters or no filters, whether rate ($P < 0.001$) or peak values ($P < 0.001$) were analyzed. Cytochalasin B (CB) treatment reduced peak chemiluminescence with IgG-coated filters by 44% ($P < 0.001$).

suspend and incubate 5×10^6 PMNs in a polypropylene tube at 37°C for 60 min. Buffer preincubated with dishes did not stimulate O_2^- release to a greater extent than did buffer from tubes (0.121±0.033 and 0.125±0.017 nmol/min, respectively, mean±SD, three experiments). (*b*) Polystyrene petri dishes processed to encourage cell adherence, spreading, and long-term growth (Corning Glass Works, Oneonta, N. Y., catalog no. 25000; Falcon Plastics, catalog no. 3001) stimulated even greater O_2^- release (0.511±0.270 nmol/min) than did the dishes used in our experiments (0.311±0.168 nmol/min, five paired experiments). LDH release from cells contacting tissue-culture dishes (1.6%) was slightly less than that from cells in contact with the standard polystyrene dishes (2.0%, means of two experiments).

DISCUSSION

During phagocytosis PMNs consume oxygen from the surrounding milieu and convert it, probably first, to O_2^- (10, 12), and then to H_2O_2 (19). Oxidation of glucose through the hexose monophosphate (HMP) shunt is stimulated, perhaps as a result of increased H_2O_2 generation (22). H_2O_2 and O_2^- may interact to form hydroxyl radicals (10, 23). The spontaneous dismutation of O_2^- is believed to result in singlet oxygen formation (reviewed in reference 10), and the occurrence of this reaction in neutrophils is considered to be the most likely explanation for the luminescence that occurs with phagocytosis (10, 21).

It was reported in 1967 that the surface-active agents digitonin and deoxycholate could stimulate in PMNs a burst of oxygen consumption and HMP shunt activation qualitatively similar to that seen during phagocytosis (24). Later studies have shown that antileukocyte antibodies, phospholipase C, the complement-derived chemotactic factor C5a, kallikrein, and phorbol myristate acetate, the active ingredient of croton oil, can induce PMNs to undergo oxygen consumption (25, 26), HMP shunt activation (25–29), and enhanced reduction of nitroblue tetrazolium dye (26, 29). Thus, it seemed possible to us that contact of PMNs with fixed surfaces in a model of immune complex disease (6–9, 30) might lead to elaboration of O_2^-, H_2O_2, and, perhaps, other toxic oxygen metabolites that could inflict tissue damage. The results indicate that leukocytes allowed to settle on polystyrene petri dishes, micropore filters, or, in particular, filters with attached aggregated IgG are induced to release O_2^- and H_2O_2 and to emit luminescence. These findings extend the observation of Henson and Oades that the HMP shunt is activated by neutrophils reacting with aggregated γ-globulin on nonphagocytosable surfaces (30).

Our findings regarding O_2^- release on surface stimulation of PMNs are in agreement with recent reports in which cells in suspension were studied. A soluble substance derived from preincubation of serum and bacteria has been reported to increase O_2^- generation by leukocytes (31); and C5a, opsonized zymosan, and heat-aggregated IgG have been shown to have a similar effect on suspended cytochalasin B-treated PMNs (32). Endotoxin and a supernate from mitogen-stimulated lymphocytes have been reported to increase superoxide dismutase-inhibitable nitroblue tetrazolium reduction by leukocytes (33), in accordance with evidence that reduction of this dye by phagocytizing PMNs depends at least in part on O_2^- formation (10, 22).

In our experiments, pretreatment of the PMNs with cytochalasin B significantly reduced generation of O_2^-, H_2O_2, and chemiluminescence. On the other hand, such pretreatment increased release of granular β-glucuronidase, as shown by others (reviewed in reference 34). This finding may be inconsistent with the concept that the phagocytic burst of oxidative metabolism is mediated by granule-associated NADPH oxidase (35), but this remains to be proven. In the least, it appears that neutrophil oxidative metabolism and degranulation can vary independently, in agreement with experiments using this same system which showed that neutrophil HMP shunt activity could be inhibited by cytochalasin B or certain agents or conditions that stimulated or had no effect on release of granular β-glucuronidase (30) and experiments with PMNs in suspension showing that soluble aggregated γ-globulin (30) or C5a (32) stimulated shunt activity or O_2^- release, respectively, but not degranulation.

The reduction in O_2^- release, as well as HMP shunt activation (30), H_2O_2 generation and chemiluminescence, by pretreatment of cells in our system with cytochalasin B contrasts with the enhanced O_2^- release from stimulated cytochalasin B-treated leukocytes studied in suspension, as recently reported by other investigators (32, 36). A likely explanation for this difference lies in the contrasting assay systems. Interference with PMN microfilament function by pretreatment with cytochalasin B would be expected to decrease pseudopod formation, spreading, and surface-cell contact (37), and thereby, stimulation of oxidative metabolism, in our system. Contact between cells and particles in suspension, however, should not be reduced by microfilament dysfunction. Moreover, ingestion and phagocytic vacuole formation would be inhibited by cytochalasin B (37), which might allow more O_2^- to be generated at the surface of the cell where it would be unavailable to intracellular superoxide dismutase (32). Thus, more O_2^- might be released to the outside where it is detected by its reduction of ferricytochrome *c*.

PMNs incubated with polystyrene petri dishes, especially those treated for tissue-culture purposes, were stimulated to produce O_2^- to a significantly greater ex-

tent than cells suspended in tubes. The dishes did not contain endotoxin or other material which could be removed by incubation with buffer, indicating that contact of PMNs with the dish surface was required for cell stimulation. Whatever the mechanism of PMN activation during incubation with polystyrene dishes, it seems important to note that such incubation does not constitute a truly "resting" or metabolically inactive state, as has often been assumed in the past.

The most stimulatory surface for PMNs in our experiments was heat-aggregated IgG bound to a micropore filter. Presumably, the cell binds to this surface through its membrane receptors for the Fc region of the immunoglobulin molecule (reviewed in reference 32). This system has been used extensively as a model for study of the pathogenesis of inflammation in diseases such as rheumatoid arthritis and certain types of nephritis believed to result from the deposition of immune complexes on tissue membranes (6–9, 30). In this model, PMN granules rupture to the outside of the cell. It would seem likely that the hydrolytic enzymes released from PMN granules could damage tissues in areas of inflammation.

It also seems likely, however, that PMN-generated O_2^-, H_2O_2, hydroxyl radical, and, perhaps, singlet oxygen could damage tissue in inflammatory sites. This might occur through destruction of thiol groups, through peroxidative decomposition of essential membrane-associated fatty acids, and through reaction with nucleic acid bases (23, 38, 39).

In support of this concept, it has been shown that superoxide dismutase or catalase can prevent depolymerization of synovial fluid by hydroxyl radicals derived from O_2^- and H_2O_2 (40), that injected superoxide dismutase apparently ameliorates inflammation (41–43), and that PMNs from patients receiving anti-inflammatory corticosteroid therapy may have decreased capacity to reduce the dye nitroblue tetrazolium (44, 45), a reaction that depends upon the conversion of oxygen to O_2^- and, perhaps, other metabolites (10).

ACKNOWLEDGMENTS

We are grateful to Dr. Edwin A. Bradley for the statistical analyses and to Dr. Ira M. Goldstein, New York University Medical Center, for helpful suggestions.

This work was supported by U. S. Public Health Service grants AI 10286, CA 13148, and CA 16673.

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