Complement and Immunoglobulins Stimulate Superoxide Production by Human Leukocytes Independently of Phagocytosis

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ABSTRACT Human peripheral blood polymorphonuclear leukocytes, when exposed to appropriate stimuli, generate significant amounts of superoxide anion (O_2^-), a highly reactive molecule which is possibly involved in bacterial killing. Since the subcellular localization and mechanism of activation of O_2^- generating systems are unknown, we have investigated superoxide dismutase-inhibitable cytochrome *c* reduction (attributable to O_2^-) by, and lysosomal enzyme release from, normal polymorphonuclear leukocytes and cells rendered incapable of ingesting particles by treatment with cytochalasin B. Neither phagocytosis nor lysosomal degranulation were prerequisites for enhanced O_2^- generation. Cytochalasin B-treated cells exposed to (*a*) serum-treated zymosan, a C3b receptor stimulus; (*b*) heat aggregated human IgG, an Fc receptor stimulus; and (*c*) the complement component, C5a, generated enhanced amounts of O_2^- in a time and concentration-dependent fashion. These cells also responded by releasing lysosomal enzymes, but there was no correlation between the ability of any immune reactant to provoke enzyme release and its ability to stimulate O_2^- generation. The three stimuli also enhanced O_2^- generation by normal (untreated) polymorphonuclear leukocytes, but only serum-treated zymosan and aggregated IgG were capable of provoking lysosomal enzyme release from normal cells. Untreated zymosan and native IgG neither stimulated O_2^- production nor provoked lysosomal enzyme release. Since enhanced O_2^- production was stimulated by immune reactants in the absence of phagocytosis, the O_2^- generating system is very likely associated with the external plasma membrane of the polymorphonuclear leukocyte. Leukocyte membrane receptors for complement and immunoglobulins may therefore not only serve in particle recognition but also may initiate biochemical events which accompany phagocytosis and killing.

INTRODUCTION

Human peripheral blood polymorphonuclear leukocytes (PMN)[1] generate superoxide anion (O_2^-) when exposed to appropriate phagocytosable and nonphagocytosable stimuli (1–6). Coincident with the ingestion of polystyrene latex particles, for example, the amount of O_2^- generated accounts for a substantial proportion of the oxygen consumed by PMN (5). This highly reactive molecule may be involved in bacterial killing (6–8) either directly or via metabolic intermediates such as singlet oxygen (9), hydroxyl radicals (10), or hydrogen peroxide (11). The nature of the O_2^- generating system, its subcellular localization in PMN, and the mechanisms whereby its activity may be enhanced, however, are unknown. An attractive hypothesis is that the O_2^- generating system is associated with the external surface

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[1] *Abbreviations used in this paper:* agg IgG, heat-aggregated human IgG; O_2^-, superoxide anion; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocytes; STZ, serum-treated zymosan.

of the PMN plasma membrane and is responsive to various particulate and nonparticulate stimuli (4).

We have investigated this possibility by measuring superoxide dismutase-inhibitable cytochrome *c* reduction (attributable to O_2^- generation) by, and release of granule associated (lysosomal) enzymes from, normal PMN and cells rendered incapable of ingesting particles by treatment with cytochalasin B (12, 13). As stimuli, we have selected serum-treated zymosan, heat aggregated human IgG, and the low molecular weight, soluble complement component C5a. Serum-treated zymosan can bind to human PMN via "specific" receptors for the opsonic fragment of the third component of complement which coats these particles (14, 15). The reactive site on aggregated IgG appears to reside on the Fc region which can engage and perturb lipid membranes (16). Aggregated IgG may also react with more specific receptors on the cell surface (17, 18). C5a, as we have previously demonstrated, interacts with PMN to stimulate their metabolism (19). All three immune reactants are capable of provoking the release of granule-associated enzymes from these cells (20–24). We have found that neither phagocytosis nor lysosomal degranulation were prerequisites for enhanced O_2^- generation by PMN.

METHODS

Preparation of leukocyte suspension. Leukocyte suspensions containing approximately 85% PMN were prepared from heparinized venous blood (10 U/ml) obtained from healthy adult donors by employing standard techniques of dextran sedimentation and hypotonic lysis of erythrocytes (20). In some experiments purified preparations of PMN were obtained by means of Hypaque/Ficoll gradients (25), allowing studies of cell suspensions containing 98±1% PMN without contaminating platelets or erythrocytes. The cells were suspended in a buffer consisting of 138 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.6 mM $CaCl_2$, and 1.0 mM $MgCl_2$, pH 7.4. This buffer was used throughout unless otherwise indicated. The osmolality of the buffer was within 5% of 300 mosmol by freezing point depression technique. Aliquots of the cell suspensions were dispensed into 10 × 75-mm polypropylene tubes (Falcon Plastics, Division of BioQuest, Oxnard, Calif.) before the addition of appropriate compounds and stimuli. Some cells were preincubated with cytochalasin B (5.0 μg/ml) (ICI Research Laboratories, Alderley Park, Cheshire, England) in 0.1% dimethyl sulfoxide (Matheson, Coleman, and Bell, East Rutherford, N. J.) at 37°C for 10 min before addition of appropriate compounds and stimuli. This concentration of dimethyl sulfoxide did not influence cytochrome *c* reduction, enzyme release, or enzyme assays (see below).

Immune reactants. Zymosan (Nutritional Biochemicals Corp., Cleveland, Ohio) was boiled and washed with 140 mM NaCl and then incubated with fresh human serum at a concentration of 10 mg/ml for 30 min at 37°C. After centrifugation and washing twice, this preparation of serum-treated zymosan (STZ) was suspended in buffer at a concentration of 5.0 mg/ml. Washed, but otherwise untreated zymosan, at an identical concentration in buffer, was used in some experiments. STZ in buffer (but not untreated zymosan) was readily agglutinated by rabbit antibody to human C3 (Behring Diagnostics, Somerville, N. J.) confirming that fragments of C3 were indeed bound to the zymosan particles (15).

C5a was generated in fresh human serum containing 250 mM epsilon aminocaproic acid (Sigma Chemical Co., St. Louis, Mo.) by adding zymosan (1.0 mg/ml) (21, 26). After 15 min of incubation at 37°C, the zymosan-serum suspension was centrifuged at 3,000 × *g* for 10 min. The particle-free supernate (2.0 ml) was then chromatographed in a 2.6 × 90-cm column of Sephadex G-75 (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) employing phosphate (10 mM)-buffered 140 mM NaCl, pH 7.4, containing 50 mM epsilon aminocaproic acid as the eluant. Filtration was performed at 4°C at a rate of 15 ml per h, and fractions of 5.0 ml were collected. These fractions were assayed for C5-derived lysosomal enzyme-releasing activity, as previously described (21), and those with peak activity were employed in the experiments described below. Appropriate volumes of eluant buffer were used as controls. The bulk of evidence regarding the identity of the lysosomal enzyme-releasing activity obtained by these methods indicates that it is a low molecular weight (approximately 17,000 daltons) product of C5, probably C5a (21). Chromatography on Sephadex G-75 of zymosan-treated serum, endotoxin-treated serum, and trypsinized human C5 yield similar low molecular weight fractions containing enzyme-releasing activity that is inhibitable by antibodies to human C5, but not by those to human C3 (21). Furthermore, the activity is resistant to heat (56°C for 30 min), is obtained in enhanced yields from serum containing epsilon aminocaproic acid, and is chemotactic for human PMN (21). These properties are identical to those of C5a. The protein content of the fractions was determined by the method of Lowry et al. (27).

IgG was isolated from fresh human serum after precipitation with 37% ammonium sulfate, desalting on a column of Sephadex G-25, and treatment with DEAE-Sephadex A50 (28). This preparation of IgG, when reacted with either rabbit antibody to whole human serum or antibody to human IgG (Behring Diagnostics, Somerville, N. J.) at a concentration of 3.0 mg/ml, yielded single precipitin bands in immunoelectrophoresis. The IgG was either aggregated by heating at 63°C for 10–30 min (agg IgG) (24) and suspended in buffer at a concentration of 3.0 mg/ml or employed in the "native" state after centrifugation at 105,000 × *g* for 30 min (16).

Determination of O_2^- generation. Duplicate reaction mixtures containing leukocytes (approximately 2–4 × 10^6 PMN) with or without cytochalasin B were incubated with appropriate compounds and stimuli in a final volume of either 1.0 or 1.1 ml for various times in the presence of 75 μM horse heart ferricytochrome *c*, Type III (Sigma Chemical Co., St. Louis, Mo.). Incubations were terminated by placing the tubes in ice, after which they were centrifuged at 4°C for 10 min at 755 × *g*. Cell-free supernates were decanted and either kept in ice or stored at −20°C before being assayed. For the determination of the amount of reduced cytochrome *c* generated during the incubations, 0.2 ml of supernate was diluted with 2.2 ml of phosphate buffer, pH 7.4, and the absorbance spectrum was measured from 540–560 nm in an Aminco, Model DW2, recording spectrophotometer (American Instrument Co., Silver Springs, Md.). With the aid of ferricyanide and dithionite, the amount of cytochrome *c* that was reduced and the total amount of cytochrome *c* present were calculated using an absorbance coefficient of 21.1 mM^{-1} cm^{-1} at 550 nm (reduced-oxidized) (5, 29). Specificity of cytochrome *c* reduction was checked by assaying supernates from reaction mixtures containing 10 μg/ml superoxide dismutase (Truett Laboratories, Dallas, Tex.) in addition to appropriate compounds and stimuli. Cytochrome *c* reduction in reaction mixtures without cells was also measured and found not to be significantly influenced by any of the other reagents. O_2^- generation is expressed as nanomoles cytochrome *c* reduced per 10^6 PMN.

Measurement of lysosomal enzyme release. The extracellular release of PMN granule-associated enzymes was measured, as previously described (20–23), in duplicate reaction mixtures identical to those employed for the determination of O_2^- generation, but in the absence of cytochrome *c*. After incubation the reaction mixtures were centriguged in the cold (755 × *g* for 10 min) and cell-free supernates removed for enzyme assays. Beta glucuronidase (EC 3.2.1.31) was determined after 18 h of incubation with phenolphthalein glucuronidate (Sigma Chemical Co.) as substrate (30). Lysozyme (EC 3.2.1.17) was determined by the rate of lysis of *Micrococcus lysodeikticus* (Worthington Biochemical Corp., Freehold, N.J.) measured by decrease in absorbancy at 450 nm (31). Crystalline egg-white lysozyme (Worthington Biochemical Corp.) was used as a standard. The cytoplasmic enzyme, lactate dehydrogenase (EC 1.1.1.27), was measured by the method of Wacker et al. (32) and used as an indicator of cell viability (20–23). Under no circumstances was there significant extracellular release of this enzyme in the experiments described below. Enzyme release is expressed as the percent of total activity released by 0.2% Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.) in simultaneously run duplicate reaction mixtures. As detailed in previous studies (20–23), appropriate control experiments (*a*) indicated there was no preferential degradation of enzyme activity in resting or treated cells and (*b*) excluded the possibilities that particles or other test reagents interfered with enzyme assays or (*c*) that there was selective absorption of enzymes to cells or particles after their release into the suspending buffer.

Other compounds. Phorbol myristate acetate (PMA) (Consolidated Midland Corp., Katonah, N. Y.) was dissolved in dimethyl sulfoxide and diluted in buffer to desired concentrations. The final concentration of dimethyl sulfoxide never exceeded 0.1%.

RESULTS

PMN O_2^- generation. PMN incubated for 15–30 min in buffer alone reduced minimal amounts of cytochrome *c* (1.4±0.2 nmol/10^6 PMN per 15 min by normal cells as compared to 1.2±0.1 nmol by cells preincubated with cytochalasin B, $n = 10$). Cytochrome *c* reduction never exceeded 0.5 nmol/10^6 PMN per 15 min in the presence of superoxide dismutase (10 µg/ml) and generally was in the range of from 0.2–0.4 nmol. These results are quite similar to values previously reported from experiments in which comparable numbers of cells and amounts of cytochrome *c* were present in reaction mixtures (3, 6).

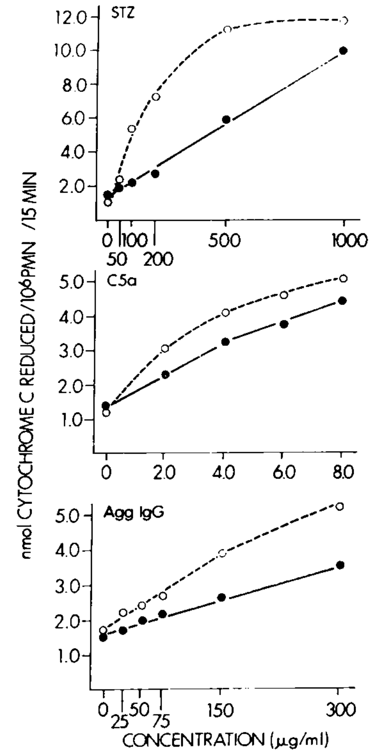

FIGURE 1 Superoxide generation by human PMN vs. concentration of immune reactants. Normal PMN (● —— ●) or PMN preincubated for 10 min with 5.0 µg/ml of cytochalasin B (O - - - O) were exposed to (A) STZ; (B) C5a; or (C) agg IgG for 15 min at the concentrations indicated. Superoxide generation is expressed as nanomoles cytochrome *c* reduced per 10^6 PMN in supernates of reaction mixtures after centrifugation at 755 × *g* for 10 min at 4°C. Results of experiments performed in the presence of superoxide dismutase (10 µg/ml) are shown in Table I.

Enhanced cytochrome *c* reduction was observed when PMN were incubated with either STZ, C5a, or agg IgG. Enhancement was, in each instance, dependent upon the concentration of the immune reactant and the duration of incubation. Examples are shown in Fig. 1 and 2. As was in the case with resting cells, cytochrome *c* reduction provoked by the immune reactants was almost completely inhibited by 10 µg/ml superoxide dismutase (never exceeding 0.8 nmol/10^6 PMN per 15 min) suggesting, therefore, that reduction was mediated by O_2^-. As in previous studies (2, 5), nearly maximal O_2^- generation occurred during the first 15 min of exposure of cells to either phagocytosable or nonphagocytosable stimuli. In general, cells rendered incapable of ingesting particles by treatment with cytochalasin B generated more O_2^- (and at a faster rate) than did untreated cells when exposed to each of the immune reactants. The greatest enhancement of O_2^- generation was observed in cytochalasin B-treated PMN exposed to STZ (Table I). STZ adhere to these cells but are not engulfed by them (33). Somewhat less O_2^- was generated by normal cells which would be expected to phagocytize these particles. A similar effect was noted with agg IgG, but much less so with the soluble stimulus, C5a.

Neither untreated zymosan particles, which do not adhere to PMN, nor "native" IgG significantly enhanced O_2^- generation by normal or cytochalasin B-treated cells. Results identical to those described

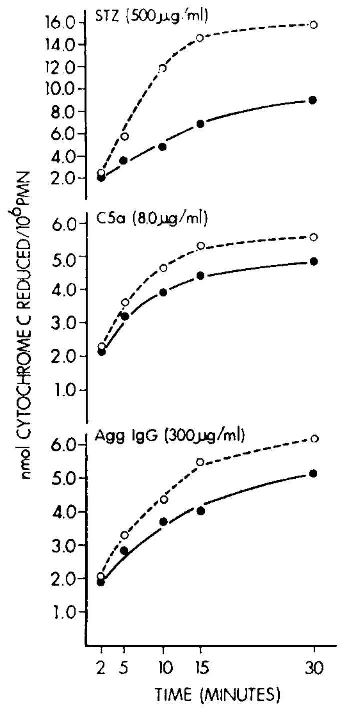

FIGURE 2 Superoxide generation by human PMN vs. duration of incubation with immune reactants. Normal (●——●) or cytochalasin B-treated (O- - -O) PMN were exposed to (A) STZ (500 μg/ml); (B) C5a (8.0 μg protein/ml); or (C) agg IgG (300 μg/ml) for times indicated.

above were obtained in three experiments employing cell suspensions which contained 98±1.0% PMN (from Ficoll-Hypaque gradients), thus excluding the possibility that cells other than PMN in the mixed-cell suspensions contributed significantly to the amounts of O_2^- measured.

TABLE I

Superoxide Generation by Human PMN

Additions	(*n*)	Cytochrome *c* reduced	
		Normal PMN	Cyto B-PMN*
		nmol/10⁶ PMN/15 min	
Buffer (controls)	(10)	1.4±0.2	1.2±0.1
Plus superoxide dismutase (10μg/ml)	(7)	0.2±0.1	0.2±0.1
Serum-treated zymosan (500 μg/ml)	(10)	7.2±0.8‡	12.2±1.1‡
Plus superoxide dismutase (10 μg/ml)	(4)	0.3±0.1	0.2±0.1
C5a (8 μg protein/ml)	(6)	4.6±0.5‡	5.1±0.3‡
Plus superoxide dismutase (10 μg/ml)	(4)	0.2±0.1	0.2±0.1
Aggregated IgG (300 μg/ml)	(6)	3.3±0.2‡	4.8±0.5‡
Plus superoxide dismutase (10 μg/ml)	(4)	0.3±0.1	0.3±0.1
Untreated zymosan (500 μg/ml)	(6)	1.3±0.1	1.2±0.2
"Native" IgG (300 μg/ml)	(6)	1.5±0.2	1.7±0.3

Results are means±SEM.

* PMN were preincubated with cytochalasin B (5.0 μg/ml) for 10 min at 37°C before additions. Cells were incubated with stimuli for 15 min.

‡ *P* vs. controls < 0.001 (Student's *t* test).

PMN enzyme release. The release from PMN of granule-associated enzymes in response to the immune reactants was measured to determine if there was any correlation between O_2^- generation by these cells and other phenomena which occur as a consequence of either phagocytosis or surface stimulation. The time and concentration-dependent release of the granule-associated enzyme, beta glucuronidase, from PMN exposed to the various immune reactants is shown in Fig. 3 and 4. The kinetics of lysozyme release were similar to that of beta glucuronidase in these experiments, but in no instance was there enhanced release of the cytoplasmic enzyme, lactate dehydrogenase (Table II), indicating that there was no significant loss of cell viability. Both beta glucuronidase and lysozyme were released to a greater extent and at a somewhat faster rate from cells preincubated with cytochalasin B. These differences were most marked during the first 15 min of exposure of the cells to the various immune reactants.

It is noteworthy that, in the absence of cytochalasin B, C5a did not provoke release of enzymes, yet this

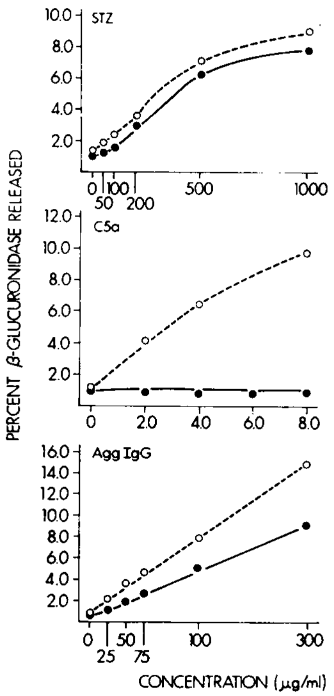

FIGURE 3 Release of beta glucuronidase from human PMN vs. concentration of immune reactants. Normal (●——●) or cytochalasin B-treated (O- - -O) PMN were exposed to (A) STZ; (B) C5a; or (C) agg IgG at concentrations indicated for 15 min. Beta glucuronidase activity in cell-free supernates is expressed as the percent of total activity released by 0.2% Triton X-100 in simultaneously run duplicate reaction mixtures (see Table II).

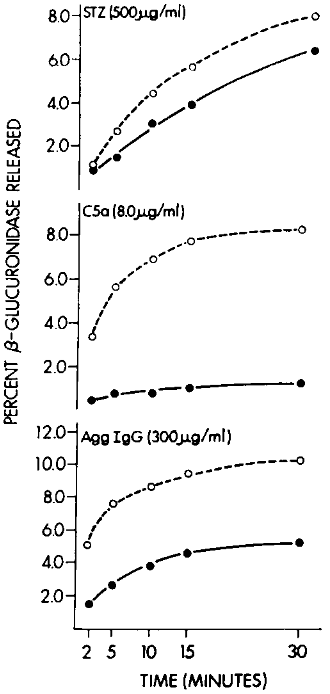

FIGURE 4 Release of beta glucuronidase from human PMN vs. duration of incubation with immune reactants. Normal (●——●) or cytochalasin B-treated (O- - -O) PMN were exposed to (A) STZ (500 µg/ml); (B) C5a (8.0 µg protein/ml); or (C) agg IgG (300 µg/ml) for times indicated.

stimulus did enhance O_2^- generation under the same experimental conditions (Table I and Fig. 1). The presence of superoxide dismutase (10 µg/ml) in the reaction mixtures did not influence release of either beta glucuronidase or lysozyme. The lack of correlation ($r = 0.04$) between the ability of the various immune reactants to provoke beta glucuronidase release and their ability to enhance O_2^- generation is demonstrated in Fig. 5A. In Fig. 5B is shown the correlation ($r = 0.97$) between release of beta glucuronidase and release of lysozyme under the same experimental conditions. Lysosomal enzyme release was maximally stimulated when cytochalasin B-treated cells were exposed to either C5a or agg IgG; however, under identical conditions O_2^- production by these cells was stimulated only to a modest degree.

Not only did the extent of enzyme release and of enhanced O_2^- generation differ in response to the various immune reactants, but the kinetics of these phenomena were also quite dissimilar (see Fig. 2 and 4). Cells exposed to untreated zymosan or to "native" IgG were neither stimulated to release enzymes nor to enhance the production of O_2^- (Tables I and II). To determine what influence released lysosomal constituents had upon cytochrome *c* reduction, cell-free supernates from appropriate reaction mixtures were added to suspensions of PMN containing cytochrome *c* and the various immune reactants. Reduction of cytochrome *c* was unaffected under these experimental conditions, indicating that released lysosomal constituents probably did not influence the assay system employed

TABLE II

Enzyme Release from Human PMN

Additions	Percent total enzyme activity released into supernate*		
	Beta glucuronidase	Lysozyme	LDH
Buffer (controls)	0.8±0.3	1.7±0.3	1.8±0.4
Plus cytochalasin B‡	1.2±0.3	2.1±0.4	1.9±0.3
Serum-treated zymosan (500 µg/ml)	6.7±0.4§	11.8±1.2§	1.7±0.4
Plus cytochalasin B	8.8±0.6§	17.5±1.6§	2.0±0.5
C5a (8 µg protein/ml)	1.0±0.2	2.2±0.3	1.7±0.4
Plus cytochalasin B	9.3±0.3§	20.0±2.2§	1.9±0.4
Aggregated IgG (300 µg/ml)	7.5±1.3§	13.6±1.7§	2.1±0.3
Plus cytochalasin B	12.1±1.9§	22.4±2.4§	2.0±0.5
Untreated zymosan (500 µg/ml)	1.7±0.5	2.8±0.4	1.7±0.2
Plus cytochalasin B	1.6±0.6	2.9±0.6	1.9±0.3
"Native" IgG (300 µg/ml)	1.4±0.3	2.2±0.2	2.0±0.3
Plus cytochalasin B	1.8±0.5	2.8±0.5	1.8±0.5

* Expressed as percent of total (100%) activity released by 0.2% Triton X-100: 100% beta glucuronidase = 13.9±1.5 µg phenolphthalein/2 × 10^6 leukocytes per h; 100% lysozyme = 7.8±1.2 µg/2 × 10^6 leukocytes; 100% LDH (lactate dehydrogenase) = 891±84 AU/2 × 10^6 leukocytes. Mean±SEM, $n = 6$.

‡ PMN were preincubated with cytochalasin B (5.0 µg/ml) for 10 min. at 37°C before additions. Cells were incubated with stimuli for 15 min.

§ P vs. controls < 0.001 (Student's t test).

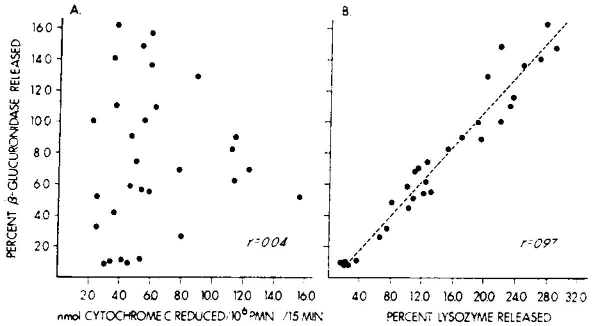

FIGURE 5 Relation between superoxide generation by, and granule-associated enzyme release from, normal and cytochalasin B-treated PMN exposed to STZ (500 µg/ml), C5a (8.0 µg protein/ml) and agg IgG (300 µg/ml) for 15 min. Results of five experiments are shown in which cytochrome *c* reduction and enzyme release were measured simultaneously. (A) Relation between beta glucuronidase release and cytochrome *c* reduction. (B) Relation between beta glucuronidase release and release of lysozyme (see Table II).

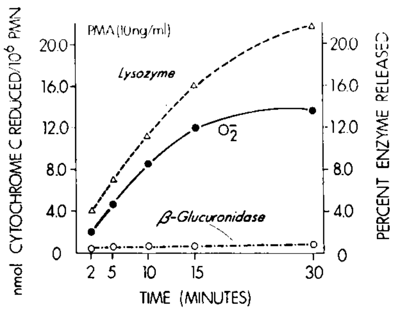

FIGURE 6 Superoxide generation by, and enzyme release from, PMN exposed to PMA vs. duration of incubation. PMN were exposed to PMA (10 ng/ml) for times indicated. Cell-free supernates were assayed for cytochrome *c* reduction (●——●), lysozyme (△- - -△), and beta glucuronidase (○-·-·○).

for the measurement of O_2^- production. Neither further reduction nor oxidation of cytochrome *c* were observed.

Response to PMA. PMA has previously been demonstrated to be a potent nonimmune stimulus for PMN enzyme release and metabolism (34, 35). The results of experiments presented in Fig. 6 and 7 indicate that this compound is also capable of stimulating PMN O_2^- generation in a time and concentration-dependent fashion. In contrast to the immune reactants, PMA provoked only the release of lysozyme and not of beta glucuronidase from these cells. Similar results have previously been reported by Estensen et al. (34).

DISCUSSION

Several investigators have recently demonstrated that superoxide dismutase inhibitable cytochrome *c* reduction (attributable to O_2^- generation) can occur in the medium surrounding intact, viable human peripheral blood PMN and is enhanced when these cells are exposed either to polystyrene latex particles or to bacteria (1–6). We have confirmed that this phenomenon also occurs when human PMN are exposed to STZ particles and agg IgG. Both particles are avidly ingested by the cells (22–24). O_2^- generation was dependent upon the duration of incubation (maximal during the first 15 min) and the concentration of immune reactants (particle to cell ratio).

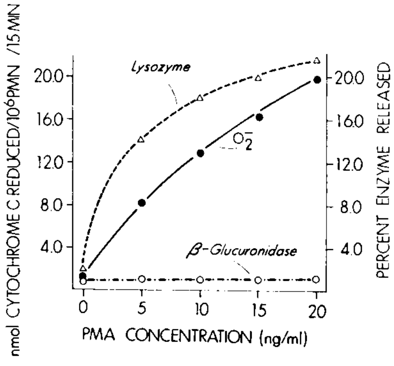

FIGURE 7 Superoxide generation by, and enzyme release from, PMN exposed to PMA (vs. concentration). PMN were exposed to PMA at concentrations indicated for 15 min. Cell-free supernates were assayed for cytochrome *c* reduction (●——●), lysozyme (△- - -△), and beta glucuronidase (○-·-·○).

Measurements of extracellular O_2^-, as performed in this and in previous studies, presumably reflect not only the production of O_2^- but also the activity of endogenous superoxide dismutase and, possibly, the rate of release of O_2^- from the cytoplasm of cells to extracellular fluid. The demonstration that human PMN contain superoxide dismutase in their cytosol (4, 6, 36) satisfied the requirement for a protective mechanism against the potential injurious effects of O_2^- and led to the proposition that O_2^- production takes place on the outer surface of the cell membrane as well as in phagocytic vacuoles which are formed by invaginations of this membrane (4). This is in accord with previous suggestions that particle-cell contact per se, and perhaps consequent structural alterations of the surface membrane of PMN, are responsible for the regulation of the metabolic behavior of these cells (37–40).

To ascertain whether O_2^- generation occurs independently of phagocytosis, we have employed the fungal metabolite, cytochalasin B, to render PMN incapable of ingesting particles. Cytochalasin B reversibly inhibits phagocytosis (12, 13), cell movement (41), and glucose transport by PMN (42), but apparently has little influence upon other cell membrane functions such as potassium and amino acid transport (43), particle binding, and dye exclusion (23, 33). Cytochalasin B-treated PMN generated enhanced amounts of O_2^- when exposed to STZ and agg IgG. In fact, the rate of production of O_2^- and the amount produced by these cells exceeded that observed when normal PMN were exposed to these same immune reactants. One explanation for this finding is that the bulk of O_2^- production occurs on the cell surface and that cytochalasin B, by inhibiting phagocytic vacuole formation, prevented access of generated O_2^- to cytoplasmic superoxide dismutase, a phenomenon which would occur by necessity if O_2^- were generated only within phagocytic vacuoles (or the cytosol) and its appearance extracellularly depended upon diffusion across the vacuolar membrane, cytosol, and the surface membrane. Cytochalasin B, therefore, may have only enhanced recovery of O_2^-.

PMN O_2^- generation was also enhanced by the soluble stimuli, C5a and PMA, in a time and concentration-dependent fashion. This is in keeping with the previously demonstrated effects of these agents upon PMN hexose monophosphate shunt activity and nitroblue tetrazolium dye reduction (19, 35, 44), effects which may be due to their interaction with cell membranes (20, 21, 34, 45). As was the case with STZ and agg IgG, the response to C5a was enhanced (but only slightly) in cytochalasin B-treated PMN. Since C5a is not phagocytosable (it is a soluble stimulus to enzyme secretion), enhanced O_2^- generation in response to C5a cannot be due to the cells' inability to form phagocytic vacuoles. Therefore, the possibility must be considered either that cytochalasin B treatment influences the "signal" provided by cell surface contact with C5a (or with STZ and agg IgG) or that cytochalasin B facilitates diffusion of O_2^- from the cytosol to the external milieu. Although cytochalasin B may have inhibited endogenous superoxide dismutase, this possibility is unlikely since appropriate experiments employing cytochalasin B and exogenous superoxide dismutase failed to reveal evidence for any interaction between drug and enzyme. Neither of the former two possibilities, however, can be excluded by the results of experiments cited in this report.

Stimulation of leukocyte O_2^- production by soluble stimuli has previously been reported. The surface-active agent, digitonin, stimulated O_2^- production by monocytic cells (46); and a factor generated by incubating serum with bacteria had similar activity with PMN (2). The identity of the latter factor is unknown, but the results reported here suggest that it may be similar to, or identical with, C5a. Other soluble cell-surface stimuli such as antineutrophil antibodies (37) and phospholipase *c* (47) have previously been shown to be capable of enhancing PMN oxygen consumption and hexose monophosphate shunt activity. It is tempting, therefore, to suggest that the effects of C5a and PMA were mediated via membrane perturbation. We cannot exclude the possibility that these agents enter the cytosol of PMN.

Inasmuch as it appeared likely that contact between the PMN surface membrane and certain immune reactants was sufficient for enhanced O_2^- generation, another aspect of this interaction was studied. Normal PMN exposed to STZ and agg IgG selectively released the granule-associated enzymes, beta glucuronidase and lysozyme, into the surrounding medium in a time and concentration dependent fashion. Such enzyme release from PMN probably occurs largely as a consequence of "regurgitation during feeding" (22), whereby fusion of lysosomal granules with incompletely closed, newly formed, phagosomes results in the leakage of granule constituents into the extracellular environment. In cytochalasin B-treated PMN, ingestion does not occur, but nevertheless, when appropriately stimulated these cells release, or secrete, lysosomal constituents by a mechanism of "reverse endocytosis" (33). Cytochalasin B, possibly by interfering with subplasmalemmal microfilaments (48, 49), facilitates membrane fusion between lysosomal membranes and the plasma membrane leading to the discharge of granule contents into the external environment as if into a phagocytic vacuole (exocytosis). This explanation is the most likely hypothesis for the enhanced release of beta glucuronidase and lysozyme from cytochalasin B-treated PMN exposed to STZ and agg IgG and the release, per se, of enzymes from such cells exposed to C5a (21). However, release of beta glucuronidase in response to STZ, agg IgG, and C5a showed no significant correlation with O_2^- generation. This was most evident with C5a, which, in the absence of cytochalasin B treatment, stimulated PMN to generate O_2^- without provoking any enzyme release. This suggests firstly, that these two phenomena are mediated by independent membrane "signals"; secondly, that fusion of granule membranes with plasma or phagosomal membranes is not a prerequisite for O_2^- generation; and finally, that "release" of O_2^- is not coincidentally linked to extrusion of lysosomal constituents. Exposure of normal or cytochalasin B-treated PMN to untreated zymosan particles or to "native" (unaggregated) IgG did not result in either O_2^- generation or enzyme release.

Particle recognition and phagocytosis by PMN are mediated in a large part by "receptors" on the cell surface for a fragment of C3 (14, 40, 50), provided by serum-treated, but not by untreated, zymosan particles (15, 51). Recognition and phagocytosis are also mediated by Fc regions of some IgG molecules that have undergone a conformational change either as a result of combining with antigen or as a result of aggregation by heating (16–18). In the case of C5a, its recognition, and as yet unknown intracellular mechanisms, result in PMN chemotaxis and granule extrusion (21). The results of the studies reported here suggest that, in addition to provoking phagocytosis, directed migration, and degranulation, the interactions between immune reactants and their "receptors" on the cell surface may generate signals which affect leukocyte metabolism.

Immune or nonimmune (e.g. PMA) stimuli may launch intracellular (cytoplasmic) events by generating soluble "mediators" at the cell surface which can then diffuse into the cytosol to provide appropriate signals and thereby exert their influence upon the intact cell. There is reason to suspect that O_2^- may be such a "mediator". For example, it has recently been reported that O_2^- generation by dialuric acid, in the fluid phase surrounding PMN, resulted in stimulation of hexose monophosphate shunt activity in these cells (52). H_2O_2 has been shown to have a similar effect (53) and is a potential product of O_2^- after spontaneous dismutation or reaction with superoxide dismutase

(4, 6, 36). Indeed, O_2^- may be generated from intact PMN by means of the recently described NADH oxidase system which is localized to the external surface of the plasma membrane and which apparently can be activated upon exposure of cells to phagocytosable particles (54, 55). Whereas our findings do not conclusively prove that a PMN ectoenzyme is capable of generating O_2^- in response to surface stimuli, they do provide evidence which supports this possibility: (*a*) O_2^- is generated by intact cells in response to surface stimulation; (*b*) O_2^- recovery is enhanced if phagocytic vacuole formation is inhibited by cytochalasin B; and (*c*) O_2^- does not appear to be released as a consequence of degranulation or cell disruption. In a recent study employing disrupted PMN, O_2^- generating activity was found to be associated with a membrane fraction (6). Surface generation of O_2^- (and H_2O_2) would allow for its concentration within phagocytic vacuoles and provides a convenient explanation for the extracellular recovery of this highly reactive molecule without having to account for its ability to diffuse from cells, containing superoxide dismutase (and catalase), without producing lethal membrane damage. Furthermore, via conversion to freely diffusable H_2O_2, extracellular or intraphagosomal O_2^- can mediate biochemical events which ordinarily accompany particle contact, phagocytosis, and bacterial killing.

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