A Role for Sulfhydryl Groups in Granulocyte Aggregation

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Polymorphonuclear leukocytes (PMN) stimulated by high concentrations of the complement component C5A form cellular aggregates, and both the rate and degree of aggregation are influenced by changes in the PMN plasma membrane and cytoskeleton. Since sulfhydryls are important constituents of the plasma membrane and cytoskeleton, we investigated the effect of agents that oxidize and bind sulfhydryls on C5A-induced aggregation. PMN incubated with diamide, a nonspecific sulfhydryl-oxidizing agent, had a marked increase in their aggregation response to C5A. Tertiary butyl hydroperoxide (BHP), which reacts specifically with the soluble sulfhydryl glutathione (GSH), had no effect on aggregation. The enhancement of PMN aggregation by diamide, but not BHP, suggested that oxidation of non-GSH sulfhydryls contributes to the aggregation response. To test the requirement for sulfhydryls in PMN aggregation, PMN were treated with the sulfhydryl-binding agent *N*-ethylmaleimide (NEM). NEM markedly impaired aggregation without affecting resting or methylene blue-stimulated [^{14}C]-L-glucose oxidation of the granulocytes. P-chloromercuriphenyl sulfonic acid (PCMPSA), an external sulfhydryl-binding agent, had no effect on aggregation. These studies suggest that cellular sulfhydryls are required for optimal PMN aggregation and that oxidation of these sulfhydryls may be one of the biochemical changes that contributes to aggregation.

HIGH CONCENTRATIONS of the chemotactic fragment of complement C5A induce several changes in polymorphonuclear leucocytes (PMN), which include reduction in their negative surface charge, formation of pseudopods from the plasma membrane, and increased stickiness of the cells to vascular surfaces and each other.[1-3] This latter effect—augmentation of granulocyte adhesiveness and aggregation—appears to be important in the early stages of acute inflammation and may contribute to the tissue damage seen in shock lung.[4] In vitro studies on PMN aggregation suggest that optimal aggregation requires calcium, magnesium, and adenosine triphosphate (ATP) production via anaerobic glycolysis.[1,5,6] They also indicate that both the PMN plasma membrane and the cytoskeleton may be involved in this response.[7,8]

Sulfhydryl groups are important constituents of the plasma membrane and cytoskeleton and may be involved in several PMN functions.[9,10] For example, substances that bind intracellular sulfhydryls have been shown to inhibit chemotactic receptor binding as well as chemotaxis.[11] In addition, oxidation of sulfhydryls may make PMN more sensitve to the effects of reactive oxygen species, with subsequent impairment of function.[12] In this study, we evaluated the importance of sulfhydryl groups in granulocyte aggregation by examining the effect of both sulfhydryl-oxidizing agents and sulfhydryl-binding agents on C5A-induced aggregation.

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MATERIALS AND METHODS

Preparation of Granulocytes

Venous blood was obtained from normal volunteers and a patient with chronic granulomatous disease (CGD) of childhood. Granulocytes were isolated by dextran sedimentation and Ficoll-Hypaque centrifugation.[13] Hypotonic shock lysis eliminated the remaining erythrocytes. Cells were washed twice and resuspended in Hanks' balanced salt solution (HBSS), free of Ca^{2+} and Mg^{2+}, with 0.5 g/dl human albumin at a final concentration of 10^7/ml. For those experiments where the sulfhydryl-oxidizing agent tertiary butyl hydroperoxide (BHP) was used, the cells were suspended in phosphate-buffered saline (PBS). The final cell preparation contained greater than 90% PMN and less than 3 platelets/100 cells. Most granulocyte preparations were used immediately after separation, but in several experiments, they were allowed to stand on ice for 3–4 hr. These latter preparations were designated "aged cells."

Preparation of C5A

Normal venous blood type AB was allowed to clot for 45 min, and the serum was removed. Epsilon amino caproic acid (32.5 mg/ml of serum) was added, and the serum was incubated in a 37°C water bath for 45 min. Zymosan (Sigma Chemical Co, St. Louis, MO) was added at a concentration of 16 mg/ml of serum. The suspension was incubated for an additional 30 min. The serum was then rendered particle-free by centrifugation at 15,000 *g* for 10 min.

Reagents

Diamide, *N*-ethylmaleimide (NEM), *P*-chloromercuriphenyl sulfonic acid (PCMPSA), and tertiary butyl hydroperoxide (BHP) were purchased from Sigma Chemical Co. Each reagent was made up in HBSS or PBS, free of Ca^{2+} and Mg^{2+}, pH 7.5. Diamide and BHP were incubated with granulocytes for 10 min at 37°C, and PCMPSA and NEM were incubated with PMN for 30 min at 37°C prior to initiating aggregation.

Granulocyte Aggregation

Granulocyte aggregation was performed using a modification of the methods of Craddock et al.[3] A standard platelet aggregometer (Payton) with dual pen recorder was used. To a siliconized cuvette, containing a Teflon bar revolving at 900 rpm, was added 5.0 μl of 0.1

M $MgCl_2$ and 0.15 *M* $CaCl_2$ solution. To this, 0.45 ml of the PMN suspension to be tested was added. The PMN were allowed to warm at 37°C for 2 min. Fifty microliters of zymosan-activated serum (ZAS) was then added and the resulting changes in light transmission recorded as ΔT. In some experiments, 25 nmole diamide in the appropriate buffer was added in place of ZAS. To provide the necessary amplification for a well defined aggregation wave, the aggregation recorder system was calibrated with a fresh PMN suspension diluted 50% (v/v) with HBSS. PMN aggregation during the initial 3 min was quantitated using a compensating polar planimeter (Keuffel and Esser Co., Germany) and expressed in square centimeters. Aggregation was verified by phase microscopy.

Statistics

Numerical data are expressed as the mean ±SD. The significance of the difference between any two sets of determinations was assessed with the paired Student's t test.[14]

Glucose Metabolism

Glucose metabolism and hexose monophosphate shunt (HMPS) activity was evaluated by the ionization chamber electrometer method.[15] The oxidation of [^{14}C]-1-glucose provided a measure of the HMPS and hexokinase activity. After baseline CO_2 production was established, methylene blue, in a final concentration 10^{-4} *M*, was added to obtain HMPS stimulation. The rate of [^{14}C]-1-glucose oxidation was calculated from the steady state or peak rate achieved after the addition of methylene blue.

RESULTS

Effect on PMN Aggregation by Agents That Oxidize Sulfhydryls

Granulocytes preincubated with 25 nmole diamide/10^6 cells and stimulated with ZAS showed a significant augmentation in the rate and amplitude of aggregation (Fig. 1 and Table 1). Lower concentrations were not effective in augmenting C5A-induced aggregation.

When diamide alone was added to granulocytes in the aggregometer, without preincubation, varying results were obtained. Most of the time, there was no significant change in light transmission, but occasionally, minimal changes occurred without visible aggregation seen on phase microscopy (Fig. 2). However, if granulocytes were allowed to stand for several hours prior to the addition of the diamide, a marked aggregation response was obtained (Fig. 2). If these granulocytes were stimulated by diamide in the absence of Ca^{2+} and Mg^{2+}, only minimal aggregation occurred.

In contrast to diamide, BHP, a compound also known to oxidize cellular GSH, did not augment C5A-stimulated aggregation. PMN in PBS buffer stimulated with C5A had aggregation responses of 29.6 ± 7.6 sq cm (n = 5), whereas PMN incubated with BHP, 100 nmole, for 10 min prior to C5A addition had aggregation responses of 26.9 ± 6 sq cm (n = 5, p = NS). Similar results were found when PMN were incubated with 100 nmole BHP and lower concentrations of diamide (5 and 10 nmole/10^6 cells), suggesting that these two compounds were not synergistic (Fig. 3).

Table 1. Effect of Varying Concentrations of Diamide on C5A-Induced Granulocyte Aggregation

Cell Suspension	Square Centimeters		*p* Value
Control	31.5 ± 6.4	n = 18	
Diamide 5 nmole/10^6 cells	35.3 ± 7.8	n = 8	NS
Diamide 10 nmole/10^6 cells	34.0 ± 7.6	n = 5	NS
Diamide 25 nmole/10^6 cells	50.6 ± 13.0	n = 5	<0.005

Granulocytes, 10^7/ml, were incubated with HBSS alone or with varying concentrations of diamide for 10 min at 37°C prior to stimulation with zymosan-activated serum (ZAS). The area under the aggregation curve was measured and expressed in sq cm. Controls for all the diamide experiments are averaged together. *p* Values were calculated by comparing individual controls to cells with diamide using the paired Student's t test.

n, Number of experiments.

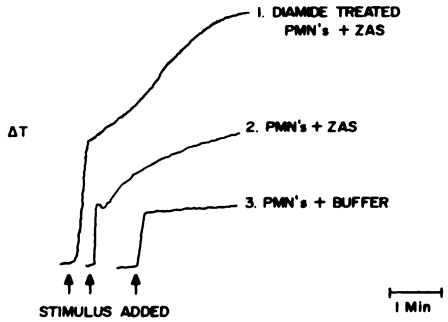

Fig. 1. Aggregation response of normal granulocytes pretreated with 25 nmole diamide (1) or pretreated with buffer (2) to zymosan-activated serum. Response of granulocytes pretreated with buffer to buffer alone (3).

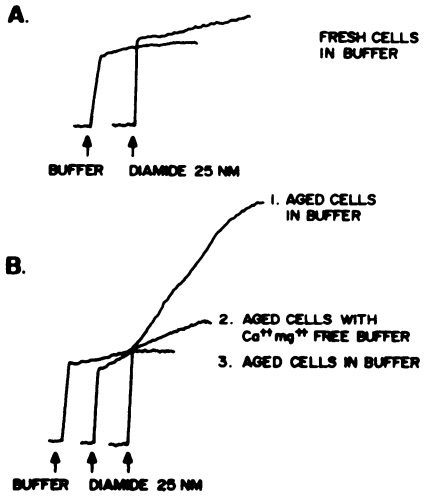

Fig. 2. (A) Aggregation response of freshly prepared granulocytes to 25 nmole diamide or buffer. (B) Aggregation response of "aged" cells to 25 nmole diamide in the presence (1) or absence of Ca^{2+} and Mg^{2+} (2). Response of aged cells to buffer alone (3).

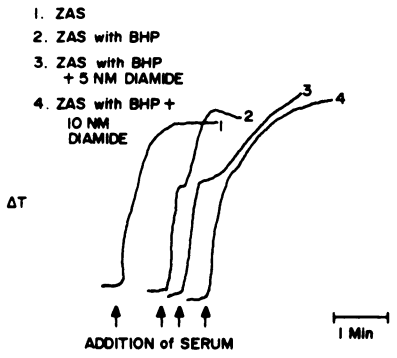

Fig. 3. Aggregation response of normal granulocytes (1), granulocytes pretreated with BHP (2), granulocytes pretreated with BHP, and 5 nmole diamide (3), or granulocytes + BHP + 10 nmole diamide (4). Zymosan-activated serum (ZAS) was used as the aggregating stimulus.

Effect on PMN Aggregation by Agents Known to Bind Sulfhydryl Groups

NEM, a cell-penetrating agent that binds both reduced glutathione (GSH) and non-GSH cellular sulfhydryls, completely prevented or markedly impaired C5A-induced aggregation (Table 2). PCMPSA, which binds to external sulfhydryls, did not alter aggregation (Table 2).

To prove that the effect of NEM was not related to a nonspecific effect, such as inhibition of glycolysis or hexose monophosphate shunt activity, we measured resting and stimulated C1 oxidation in PMN treated with NEM. Resting C1 oxidation was actually increased over controls in NEM-treated cells, and these cells responded normally to methylene blue stimulation (Fig. 4).

Studies With CGD PMNs

Aggregation studies were also done with the granulocytes of a patient with chronic granulomatous disease (CGD). Similar to normal PMNs, diamide did not induce aggregation in freshly prepared cells. However, diamide did augment the stimulation induced by C5A. The area under the aggregation curve of CGD cells induced by C5A alone was 24 sq cm, a value similar to that observed for normal PMN. CGD PMN, preincubated with diamide and then stimulated by C5A, showed almost a twofold increase, with a value of 43.5 sq cm.

Table 2. The Effect of Sulfhydryl-Binding Agents on C5A-Induced Granulocyte Aggregation

Cell Suspension	Square Centimeters		*p* Value
HBSS alone	17.2 ± 3.4	*n* = 11	
HBSS + ZAS	32.7 ± 8	*n* = 8	0.001
HBSS + ZAS + 3 nmole NEM/10^6 cells	16.8 ± 4.8	*n* = 6	0.001
HBSS + ZAS + 10 nmole PCMPSA/10^6 cells	33.4 ± 3	*n* = 4	NS

Granulocytes, 10^7/ml, suspended in HBSS were incubated (37°C for 30 min) with sulfhydryl-binding agents prior to stimulation with ZAS. *p* Values were calculated by comparing ZAS-stimulated cells to buffer alone or cells with sulfhydryl-binding agents to those without, using the paired Student's t test.

n, Number of experiments.

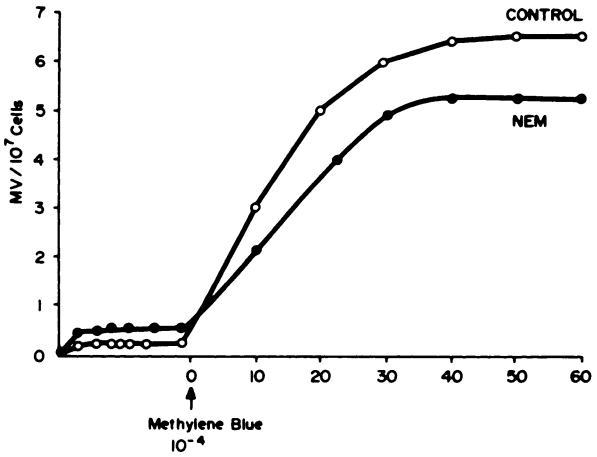

Fig. 4. The effect of 3 nmole NEM on C1 oxidation in the resting cell and after stimulation with 10^{-4} *M* methylene blue. The curves represent a continuous measurement of $^{14}CO_2$ production by granulocytes in buffer. The Y axis represents the electrical signal in millivolts generated by $^{14}CO_2$ passing through the ionization chamber. The X axis indicates time in minutes.

DISCUSSION

Polymorphonuclear leukocytes are constantly exposed to their own secretory products as well as those of other cells. These products include organic and inorganic radicals that can induce oxidant damage and may result in alteration of the cell membrane and cytoskeleton, with concomitant changes in PMN mobility and other functions.[16]

In an attempt to simulate this oxidant damage, we exposed cells to diamide, a potent thiol-oxidizing agent. The major target of diamide in PMN appears to be GSH,[9,11,17] although nonspecific oxidation of other sulfhydryl groups or cellular substrates may also occur. At the concentrations of diamide used in this study, PMN GSH levels are significantly reduced but never completely consumed.[9] In addition, PMN treated with diamide show an impairment in microtubule assembly in response to mitogenic stimulation with concanavalin A,[9] but exhibit normal membrane-related functions, including phagocytosis and the accompanying metabolic burst.[12] We tried to correlate the reported effects of diamide on GSH and microtubule function with its ability to enhance C5A-induced aggregation, but could not for the following reasons. First, BHP causes similar reductions in GSH levels and impairs microtubule assembly without affecting

aggregation.[9] Second, other microtubule inhibitors, such as colchicine and vinblastine, either impair aggregation of PMN or have no effect.[5,7]

Another mechanism by which diamide could alter aggregation is nonspecific oxidation of other cellular sulfhydryls or membrane proteins. We thus tried to distinguish between effects on membrane proteins or other sulfhydryls by further evaluating the importance of sulfhydryls in PMN aggregation. The ability of low concentrations of NEM to block aggregation implied that intact sulfhydryls are required for aggregation. Although at higher concentrations, NEM may have effects on PMN other than sulfhydryl blockade, at the concentrations used, it did not impair either chemotactic binding[11] or resting and stimulated glucose metabolism. Interestingly, other investigators have demonstrated that NEM impairs C5A-induced chemotaxis.[11] Our observation that an external sulfhydryl-binding agent (PCMPSA) did not impair aggregation suggests that the sulfhydryls involved in aggregation are internal to the membrane.

An additional finding in our study was that diamide treatment alone did not cause significant aggregation in fresh cells but did so in aged cells. Moreover, optimal aggregation in response to diamide was dependent on adequate extracellular concentrations of magnesium and calcium. This requirement for the cations is similar to other aggregating stimuli, and it implies that diamide-induced aggregation is an active process.[6] Although unusual, the differential effects of diamide on PMN preparations is not unique. For example, Boxer[18] has shown that amphoteracin B only induced aggregation in incubated cell preparations. During prolonged incubation, PMN may undergo certain changes, including loss of calcium and alterations in the surface membrane, which make them susceptible to agents that increase cell adhesiveness.[1]

Previous investigators have demonstrated a role for sulfhydryl groups in PMN adhesion, chemotaxis, phagocytosis, microtubule assembly, and binding of chemotactic factor.[9-11] We propose that they are also involved in C5A-induced aggregation. When granulocytes are stimulated by C5A, they may generate metabolites or release intracellular enzymes that oxidize sulfhydryl groups, with a resultant increase in their ability to aggregate. We originally thought that reactive oxygen species generated by PMN might be involved, but it seems unlikely, as the CGD PMN aggregated normally and radical scavengers did not affect aggregation.[19] However, both normal and CGD PMN produce other substances that may stimulate PMN aggregation. These include products of arachidonic acid metabolism, and part of their effect may be a consequence of sulfhydryl oxidation.

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