Superoxide-Dependent Chemotactic Activity for PMNs Derived from Opsonized Zymosan-Stimulated Human Platelets

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Summary

Previous studies have shown that platelets exhibit a H_2O_2 producing, NADH-dependent system that is activated by interaction with particulate material. Current evidence suggests that this system could be critically involved in the generation of chemotactic factor(s). In the present studies, chemotactic activity for polymorphonuclear leukocytes of supernatants derived from zymosan-stimulated human platelets has been evaluated using an agarose gel technique. Supernatants of opsonized zymosan-stimulated platelets showed significant chemotactic activity (migration index = 300 ± 50), in comparison with supernatants prepared from platelet suspensions stimulated with nonopsonized-zymosan (migration index = 10 ± 15) or resting platelet supernatants (migration index = 15 ± 15). Furthermore, a marked increase in chemotactic activity of the opsonized zymosan-treated platelet supernatants was demonstrated after the addition of NADH (migration index = 525 ± 100). The inclusion of specific inhibitors of the cyclooxygenase and lipoxygenase pathways resulted in a marked reduction of chemotactic activity, which was restored in the presence of NADH. Further, the addition of superoxide dismutase completely abolished the chemotactic response induced by NADH. These data suggest that platelets are the source of chemotactic factor(s) derived from the activation of a superoxide generating system.

Speculation

Platelets play an important role in the nonspecific immune mechanisms by participating in the recruitment of inflammatory cells to the site of inflammation and in the cell-to-cell interaction. This function appears to be related to the production of chemotactic factors some of which are associated with a superoxide generating system.

Previous investigations have demonstrated that human platelet suspensions generate superoxide (O_2^-) anions at rest and after aggregation (17). It is now evident that the production of oxidizing substances by platelet suspensions is not due to polymorphonuclear leukocyte (PMN) contamination (16) and/or to passive diffusion from other cells (14). Using a cytochemical, ultrastructural method, we have recently demonstrated that platelets possess an H_2O_2 producing, NADH-dependent system that is stimulated by interactions with particulate material (8).

Considerable accumulated evidence indicates that H_2O_2 , and perhaps superoxide anions, released by granulocytes play a critical role in oxygen-dependent microbicidal activity (22). Platelets, although phagocytic, do not kill ingested bacteria (5).

To date, the physiologic role of the platelet oxidizing substance(s) generating system is not completely understood. Present

evidence suggests that this system could be involved in the production of chemotactic factors. Perez *et al.* (20) suggested that an O_2^- generating system can produce chemotactic lipids from arachidonic acid. Moreover, it has been proposed that superoxide radicals play a role in cell-to-cell interaction between inflammatory cells (10, 18). In this way, generation of superoxide could provide a self-amplifying communication system by reacting with an inactive plasma component, converting it to an active, stable and potent chemotactic factor (21).

Because platelets are involved in the inflammatory response and in immunologic processes (2, 4), the present study was performed to investigate whether PMN movement could be influenced by the platelet superoxide generating system.

Previous investigations have demonstrated the role of opsonized zymosan in the stimulation of human platelets (19, 31) and in the activation of the NADH-dependent, cyanide-insensitive, oxidase system (9). We now report that supernatants of opsonized zymosan-treated platelets, after inhibition of prostaglandin pathways, exhibit chemotactic activity for PMN and that this activity can be completely abolished by superoxide dismutase (SOD).

MATERIALS AND METHODS

Preparation of platelet suspension. Purified suspension of platelets were prepared from fresh blood samples using 2% ethylene diaminetetracetic acid (EDTA, BDH) (9:1 v/v) as anticoagulant. Only those volunteers free of any drug treatment for at least 10 days were chosen. Particular care was taken to minimize leukocyte contamination (less than 1 leukocyte per 10^8 platelets). A platelet-rich preparation (PRP) was obtained by centrifugation of blood samples at $100 \times g$ for 15 min at room temperature. The supernatants were then gently layered on Ficoll (Ficoll 400, Pharmacia) and centrifuged at $80 \times g$ for 20 min. The resulting supernatants were mixed with an equal volume of Dulbecco's phosphate buffer saline (PBS, Gibco) and washed twice.

Activation of platelet suspension. Zymosan A (Sigma) was boiled in distilled water for 20 min, washed three times in 0.15 M NaCl, incubated (50 mg/ml) with autologous, citrated plasma for 30 min at 37°C and then washed three more times in Dulbecco's PBS. The PRPs were then activated by mixing with an equal volume of opsonized zymosan for 30 min at 37°C . The generation of prostaglandin products derived from the cyclooxygenase pathway was inhibited by the addition of 100 mM aspirin (ASA, Bayer). Both the cyclooxygenase and lipoxygenase pathway were inhibited by the addition of 300 $\mu\text{g}/\text{ml}$ of 5,8,11,14-eicosatetraenoic acid (ETYA, RO 3-1428, Roche). One mM KCN (Merck) was used to inhibit mitochondrial respiration and 10 $\mu\text{g}/\text{ml}$ theophylline (Sigma) were added to inhibit platelet aggregation and granule protein release. The zymosan-activated platelet suspensions were then evaluated for the generation of O_2^- anions and O_2^- -mediated

generation of chemotactic factors unrelated to prostaglandin metabolism by the addition of 0.5 mM NADH (Sigma). To inhibit the NADH-dependent oxidase system, 50 μ g/ml SOD (Sigma) was used. Heat-inactivated SOD was also employed as the control experiment. Control experiments were carried out using supernatants from: (1) resting platelets; (2) resting platelets plus inhibitors; (3) resting platelets plus inhibitors and NADH; (4) nonopsonized zymosan-activated platelets; (5) nonopsonized zymosan-activated platelets plus inhibitors; and (6) opsonized zymosan alone without platelets (tables 1 and 2). After incubation, the platelet suspensions were centrifuged at $200 \times g$ for 20 min, and the cell-free supernatants collected and used as sources of chemotactic factor(s) for PMNs.

Evaluation of chemotactic activity. Each supernatant was dialyzed against polyethylene glycol (PEG 6000, BDH), in order to concentrate the active component(s), filtered through Millipore filters (0.45 micron), and then evaluated for chemotactic activity using normal human PMNs as responding cells in an agarose assay system (6). Briefly, 1.5 ml of a 1.2% agarose (Reo 15, Boehringer) solution in RPMI 1640 (Gibco) with 10% fetal calf serum (FCS, Flow) were added to 35×10 mm tissue culture dishes. Three wells (4 mm in diameter and spaced 2 mm apart) were cut using a suitable template punch. Suspensions of PMNs were obtained from blood samples of healthy volunteers according to methods previously described (7), and used at a concentration of 30×10^6 cells/ml. Each test was performed in duplicate; 10 μ l of the cell suspension were added to the central well and 10 μ l of each supernatant or zymosan-activated serum, as positive control, were added to one of the lateral wells. The other well was filled with 10 μ l of RPMI 1640, as negative control. The dishes were

covered and incubated in a 37°C humidified atmosphere with 5% CO₂ for 3 h. Afterwards, the preparations were fixed with May-Grunwald (Hoechst) for 30 min, the agarose layers removed, and the plates stained with Giemsa (Hoechst). The migrated cells were counted, using a 10 x eyepiece grid (10 $\times$ 10 mm) at X10 magnification. The number of cells randomly migrating towards the buffer was subtracted from the number of cells migrating directly toward the attractant to obtain the net chemotactic response. Each preparation was also projected using a Leitz micro-projector (magnification scale 1:175) and the maximum distance of migration was measured and the distance of random migration was subtracted. The migration index (M.I.) was then calculated by the following formula:

$$\text{M.I.} = \frac{(N_{ca} - N_b) \times (D_{ca} - D_b)}{100}$$

Where N_{ca} , number of cells which had migrated toward the chemoattractant (ca); N_b , number of cells which had migrated toward the buffer (b); D_{ca} , the maximum migration distance toward the chemoattractant (ca); and D_b , the maximum migration distance toward the buffer.

Platelet studies. In order to verify the effect of various inhibitors on platelet metabolism and function, O₂ consumption, before and after the addition of the inhibitors, was measured polarographically with a Clark's electrode (Yellow Spring Instruments Co., Yellow Spring, OH), in the presence or in the absence of thrombin (final concentration, 1.7 U/ml).

Platelet aggregation was measured photometrically (3), using an aggregometer (ELVI).

Table 1. Chemotactic activity of stimulated and unstimulated platelet supernatants

Supernatants	M.I. (mean $\pm$ S.D.)	Number of experiments
Resting platelets	15 $\pm$ 15	4
Opsonized zymosan	12 $\pm$ 10	4
Nonopsonized zymosan-stimulated platelets	10 $\pm$ 15	4
Opsonized zymosan-stimulated platelets ¹	300 $\pm$ 50	4
Resting platelets + NADH	20 $\pm$ 15	4
Opsonized zymosan-stimulated platelets + NADH ²	525 $\pm$ 100	4

¹ $P < 0.001$ when compared with resting platelets, or nonopsonized zymosan-stimulated platelets.

² $P < 0.01$ when compared with opsonized zymosan-stimulated platelets.

Table 2. Chemotactic activity of stimulated and unstimulated platelet supernatants + inhibitors¹

Supernatants	M.I. (mean $\pm$ S.D.)	Number of experiments
Resting platelets $\pm$ inhibitors	18 $\pm$ 10	4
Resting platelets + inhibitors + NADH	12 $\pm$ 20	4
Nonopsonized zymosan-stimulated platelets + inhibitors	10 $\pm$ 5	4
Opsonized zymosan-stimulated platelets + inhibitors	10 $\pm$ 8	5
Opsonized zymosan-stimulated platelets + inhibitors + NADH ²	320 $\pm$ 100	5
Opsonized zymosan-stimulated platelets + inhibitors + NADH + SOD	25 $\pm$ 20	3
Opsonized zymosan-stimulated platelets + inhibitors + NADH + heat-unactivated SOD	300 $\pm$ 100	3

¹ ASA, ETYA, and KCN (for explanations, see text).

² $P < 0.001$ when compared to opsonized zymosan-stimulated platelets + inhibitors.

RESULTS

The results of chemotactic activity of supernatants derived from stimulated and unstimulated platelets after various treatments are shown in Table 1.

The M.I. obtained testing normal PMNs with opsonized zymosan was 522 ± 222 . This has been considered as positive control.

The supernatants of opsonized zymosan-stimulated platelets showed significant chemotactic activity (M.I. = 300 ± 50 , $P < 0.01$; Student *t* test), in comparison with supernatants prepared from platelet suspensions stimulated with nonopsonized-zymosan (M.I. = 10 ± 15), or resting platelet supernatants (M.I. = 15 ± 15). The addition of NADH resulted in a marked increase of chemotactic activity in the suspensions of opsonized zymosan-treated platelets (M.I. = 525 ± 100 , $P < 0.01$).

The results of studies designed to determine the effect of inhibitors on the chemotactic activity are shown in Table 2. It can be seen that the inclusion of specific inhibitors of the cyclooxygenase and lipoxygenase pathways, i.e., ASA and ETYA, significantly reduced the chemotactic activity of supernatants derived from opsonized zymosan-stimulated platelets (M.I. = 10 ± 8); nevertheless, the stimulated platelets still retained the capacity to generate chemotactic factor(s) in the presence of NADH (M.I. = 300 ± 100 , $P < 0.001$). This chemotactic activity was completely abolished by the addition of SOD (M.I. = 25 ± 20), whereas unactivated SOD did not modify the chemotactic activity (Table 2).

The platelet function was completely inhibited, as demonstrated by the aggregation studies; moreover, the inhibition of prostaglandin pathways by ASA and ETYA was verified by the lack of thrombin-induced oxygen consumption in the treated platelet suspensions. In control experiments, the supernatants of PMN suspensions, as well as mononuclear cells, at concentrations equivalent to any possible contamination of PRP failed to generate chemotactic activity, either in the presence or absence of NADH.

DISCUSSION

The results of the present investigations suggest that significant chemotactic activity is produced by platelets in the presence of

NADH after challenge with opsonized zymosan. Superoxide radicals were required as shown by essentially complete inhibition by SOD.

Previous studies have demonstrated that after stimulation of PMNs and monocytes by any of a variety of stimuli (e.g., particulate challenge), a membrane perturbation that is associated with NAD(P)H oxidase activation, occurs, which leads to the formation of superoxide radicals (1). Our data, showing an increase of PMN chemotactic response to stimulated platelet supernatants after the addition of NADH and its inhibition by SOD, strongly suggest that production and/or release of factor(s) with chemotactic activity for PMNs could be linked to a superoxide-generating system. The possibility that superoxide anions could be produced by contaminating PMNs can be excluded by our control experiments; moreover, previous studies from our laboratory have suggested that in purified platelet preparations, the contribution of PMNs in the production of superoxide radicals appears to be negligible (8).

Chemotactic factors are heterogeneous in type and appear to arise from widely different sources (2, 27). Under the present experimental conditions, in which the presence of exogenous factors such as bacterial products can be excluded, both cellular and humoral factors appear to be responsible for PMN chemotaxis. In the present studies, which were conducted in absence of plasma and serum, the possibility that serum derived chemotactic factors as well as those derived from coagulation and fibrinolytic systems, or from kinin-generating pathways, could also be excluded. In addition, chemotactic activity was not demonstrated with mononuclear cell contamination (up to $1 \text{ cell}/10^3$ platelets). The present study, therefore, suggests that platelets themselves generate chemotactic activity. To date, two different mechanisms of generations of chemotactic activity by platelets have been reported. Stimulation of platelets can induce release of a chemotactic factor from granules which interacts with the complement cascade (29); moreover, it has been suggested that oxygenate arachidonate products via lipoxygenase, are potent chemotactic factors (13, 15, 26). The first hypothesis could be ruled out because the experiments were carried out in the absence of serum and in the presence of platelet aggregation and release inhibitors (cyanide, theophylline, . . .). Perturbation of platelet membranes leads to mobilization of arachidonic acid from membrane phospholipids (24); the mobilized arachidonic acid may subsequently be oxidized by either cyclooxygenase and/or lipoxygenase pathways. Products of both cyclooxygenase, i.e., 12-L-hydroxy-5,8,10-heptadecatrienoic acid, HHT, and lipoxygenase pathways, i.e., 12-L-hydroxy-5,8,10,14-eicosatetraenoic acid, HETE, are potent chemotactic factors for human eosinophils and neutrophils (15, 28). Anti-inflammatory agents such as aspirin and indomethacin inhibit the cyclooxygenase pathway whereas the acetylenic compound 8,11,14-eicosatetraenoic acid (ETYA) blocks both pathways (11, 23, 30). In the present study, chemotactic activity for PMNs was still present (in the presence of NADH) when lipoxygenase and cyclooxygenase inhibitors were added to the platelet suspensions. This finding excludes the possibility that the platelet-derived chemotactic factor(s) arise from arachidonate metabolites and suggests that a superoxide-generating system is involved. It has been shown that nonenzymatic oxidation of arachidonic acid may generate biologic chemotactic factor without the activation of either lipoxygenase or complement system (25). Fridovich and Porter (12) demonstrated that hydroperoxides are formed by superoxide-mediated arachidonic acid oxidation. Their experiments provide a direct chemical link with findings reported by other investigators. Petrone *et al.* (21) found that superoxide can react with a precursor in plasma to generate a factor that is chemotactic for neutrophils. This generation was strictly superoxide-dependent because it was inhibited by superoxide dismutase, but not catalase; moreover, it has been reported that the *in vitro* incubation of purified arachidonic acid with a superoxide-generating system results in the formation of chemotactic products (20).

The present study demonstrates that platelets themselves are also capable of generating chemotactic activity in the absence of

plasma and in the presence of NADH. Because NADH does not cross the membrane, our data support the hypothesis that a NADH-dependent-oxidizing substance-generating system is present on platelet plasma membrane. Finally, it has been shown that platelet stimulation by a number of agents is associated with membrane alterations that lead to increased enzyme activities. One possible explanation of the present results is that perturbation of membranes by opsonized-zymosan activates an oxidase system, leading to the generation of superoxide radicals. These O_2^- radicals can, in turn, react with a platelet component resulting in the production of factor(s) highly chemotactic for PMNs.

In conclusion, although further investigations are necessary to better clarify the specific nature of this factor(s) and its chemical characteristics, the present studies suggest that platelets play an important role in the nonspecific immune response by participating in the recruitment of inflammatory cells to the site of inflammation. This platelet function appears to be related to the production of chemotactic factors, some of which are associated with a superoxide-generating system.

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