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Free radicals and inflammation: Superoxide-dependent activation of a neutrophil chemotactic factor in plasma

(granulocyte/leukocyte/chemotaxis/superoxide dismutase)

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ABSTRACT **The intravenous administration of superoxide dismutase (superoxide:superoxide oxidoreductase, EC 1.15.1.1) to animals with induced inflammation suppresses the inflammatory response and inhibits leukocyte infiltration into the challenged site, suggesting that neutrophil-generated superoxide reacts with an extracellular precursor to generate a substance chemotactic for neutrophils. Plasma exposed to superoxide *in vitro* becomes potently chemotactic. The appearance of chemotactic activity is inhibited by superoxide dismutase but not by catalase. The chemotactic factor does not stimulate superoxide production or degranulation in neutrophils. Intradermal injection of superoxide-treated plasma or of a superoxide-generating system causes heavy infiltration of neutrophils to the injection site but does not cause overt signs of inflammation. The chemotactic factor consists of a chloroform-extractable component bound to serum albumin. The superoxide-dependent chemotactic factor appears to play a major role in communication in neutrophil-mediated inflammatory events. Prevention of production of this factor appears to be the major anti-inflammatory action of superoxide dismutase.**

The participation of superoxide in the inflammatory response has been inferred from the anti-inflammatory effect of parenterally administered superoxide dismutase (superoxide: superoxide oxidoreductase, EC 1.15.1.1) (1, 2). Upon activation by complement (3), aggregated immunoglobulin (3), endotoxin (4), lymphokines (4), or phagocytosis of opsonized particles (5), neutrophils produce and release superoxide. This free radical or the oxidative species derived from it are toxic to cells and tissues (6–10), and this has been assumed to account for the anti-inflammatory activity of superoxide dismutase (11, 12). However, recent evidence suggests that superoxide *per se* is relatively innocuous and that the role of superoxide in inflammation could not be due solely to direct cytotoxicity (2, 9). Moreover, no profound anti-inflammatory activity was seen with hydroxyl radical scavengers or catalase (2), arguing against roles for $OH\cdot$, H_2O_2, and singlet oxygen.

Our previous report (13), as well as experiments described herein, demonstrate that superoxide dismutase inhibits the migration of neutrophils to sites of inflammatory challenge. Experiments were designed to investigate whether neutrophil mobilization results from generation of a superoxide-dependent chemotactic factor. Such a factor would provide a self-amplifying mechanism whereby the first stimulated neutrophils at the site of a potential inflammatory lesion could call additional leukocytes to the area. The data indicate that superoxide reacts directly with a plasma precursor to form an activity that is chemotactic for neutrophils.

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MATERIALS AND METHODS

Ferricyochrome *c*, CNBr, sodium xanthine, xanthine oxidase (grade III), catalase, human serum albumin (fatty acid free), bovine serum albumin (Cohn fraction V), and ovalbumin were obtained from Sigma. Xanthine oxidase was desalted by Sephadex G-25 chromatography and dissolved in 50 mM potassium phosphate buffer (pH 7.8) at a concentration of 1–2 units/ml (14). Superoxide generation and superoxide dismutase activity were assayed by the cytochrome *c* reduction assay (14). Hanks' balanced salt solution was obtained from GIBCO. Ficoll (M_r, 70,000) and dextran (M_r, 200,000) were obtained from Pharmacia. Lucite blind well chemotactic chambers were from Nucleopore (Pleasantown, CA). Three and 5-μm-pore cellulose acetate filters for chemotaxis were from Millipore.

High molecular weight derivatives of superoxide dismutase and catalase were prepared by attaching the enzymes to CNBr-activated Ficoll by a procedure based on that of Kagedal and Akerstrom (15). Ficoll (500 mg) was mixed with 150 mg of CNBr at pH 10.4. The pH was maintained by the addition of NaOH. After 30 min, the pH was adjusted to 9.0 with $NaHCO_3$, the enzyme (100–200 mg) was added, and the mixture was stirred overnight at room temperature. The solution was concentrated by ultrafiltration. The molecular weight of the derivatized enzymes was >150,000. Approximately 50% of the original enzymatic activity was retained. The circulating half-life of Ficoll–superoxide dismutase was 14–24 hr in the rat (2).

Human leukocytes and plasma were from blood of young healthy adults of both sexes. Leukocytes were obtained after the blood was mixed (1:4) with 6% dextran in saline. After the erythrocytes sedimented, the cells of the upper layer were collected, washed once with Hanks' balanced salt solution, and resuspended in this solution at 3–5 $\times$ 10^6 neutrophils per ml. Plasma was prepared from heparinized blood by centrifugation.

Superoxide production by stimulated neutrophils was measured by a modification of the method of Salin and McCord (16). Acid phosphatase release was determined as described (17).

The reverse passive Arthus reaction and carrageenan-induced foot edema test were performed with rats as described (2). The inflammatory responses were quantified by gravimetric assessment of edema. The degree of leukocyte infiltration was determined by light microscopic examination of tissues that

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were fixed, sectioned, and stained with hematoxylin and eosin.

Chemotactic activity was assessed by the Boyden method (18). For most experiments, 1–2 × 10^6 cells per chamber and an incubation time of 95 min were used. Filters were stained with hematoxylin (19) and the lower surfaces were examined microscopically. Ten fields were counted and averaged. Results are given as the mean ± SEM for replicate chambers.

Lipids were extracted from neat plasma with chloroform or chloroform/methanol, 1:1 (vol/vol). Twenty milliliters of plasma was mixed with 60 ml of the solvents with a Brinkmann Polytron. The extract was evaporated under nitrogen and the yellow residue was dissolved in 100 μl of dimethyl sulfoxide. This solution was dissolved in 2 ml of Hanks' balanced salt solution or 2 ml of a 0.4% solution of fatty acid-free human serum albumin or ovalbumin in Hanks' balanced salt solution at pH 7.2. Whereas the solutions of extracts in salt solution were cloudy, those containing protein were clear. The solutions were treated and assayed for chemotactic activity as described below.

RESULTS

Previous studies (2) of the pharmacokinetics of intravenously administered superoxide dismutase showed that the native enzyme was cleared from the circulation of the rat with a half-life of 6 min. However, high molecular weight derivatives of the enzyme consistently showed prolonged half-lives with dramatically increased anti-inflammatory activity (2). When administered to rats 1 hr prior to inflammatory challenge, the Ficoll derivative of superoxide dismutase dramatically inhibited the edema associated with the Arthus response (Table 1). Ficoll alone or a Ficoll derivative of catalase showed no anti-inflammatory activity, indicating that the inhibition was due to the presence of enzymatically active superoxide dismutase. The Ficoll derivative of the enzyme was also effective in suppressing the nonhistamine phase of the carrageenan-induced foot edema when the derivative was administered 1 hr prior to the carrageenan. Moreover, the neutrophil influx to the Arthus or carrageenan-challenged sites was markedly diminished in the animals pretreated with derivatized superoxide dismutase (Fig. 1 *A* and *B*). A Ficoll derivative of catalase, which had a long circulating lifetime, had no effect on either inflammation or leukocyte infiltration. These results suggest that, in these models of inflammation, the infiltration of neutrophils is dependent upon the generation of the superoxide radical.

Table 1. Anti-inflammatory activity of superoxide dismutase

Model	Treatment	% inhibition	Clearance half-life
Reverse passive Arthus*	Saline	—	
	Native enzyme, 24,000 units/kg	0	6 min
	Ficoll-enzyme, 24,000 units/kg	95	24 hr
Carrageenan foot edema†	Saline	—	
	Native enzyme, 24,000 units/kg	0	6 min
	Ficoll-enzyme, 4,800 units/kg	54	24 hr

All treatments were administered intravenously 1 hr prior to elicitation of the inflammatory response. Each experiment included at least seven animals.

* The reaction was elicited by intravenous injection of human serum albumin (5 mg/kg), followed by the intradermal injection of rabbit anti-human serum albumin (50 μl). Lesion weight was determined 3.5 hr later. Edema weight was determined by subtraction of the average weight of uninflamed skin punches (from animals that received antibody but no antigen). Inhibition is relative to a saline-treated control.

† Edema was assessed by amputating and weighing the feet 4 hr after injection of carrageenan.

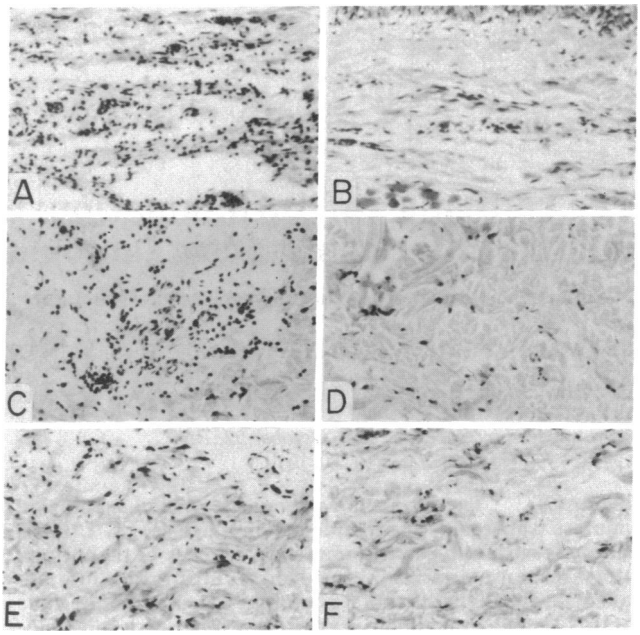

FIG. 1. Superoxide-dependent accumulation of inflammatory cells *in vivo*. (×100.) (*A* and *B*) Treatments were: (*A*) subplantar injection of 0.1 ml of a 1.5% suspension of carrageenan (Sigma, type I); or (*B*) at 1 hr prior to carrageenan injection, 24,000 units of Ficoll-superoxide dismutase per kg intravenously. Four hours after administration of carrageenan, the animals were killed and the feet were excised, fixed, sectioned, and stained. A heavy, diffuse infiltrate is evident in the untreated animal given only carrageenan.

(*C* and *D*) Rat plasma was exposed to superoxide by incubating the following at 37° for 2 hr: 0.5 ml of plasma, 0.1 ml of 1 mM xanthine, and 0.002 unit of xanthine oxidase. For control, xanthine and xanthine oxidase were allowed to react to completion before the addition of plasma. Rats were injected intradermally with 50 μl of these mixtures at shaved, depilated, laterally paired dorsal sites. At 5 hr later the animals were killed and the sites were excised, fixed, sectioned, and stained. The tissue receiving superoxide-exposed plasma (*C*) showed a heavy, diffuse infiltrate not seen in the control (*D*).

(*E* and *F*) Rats were injected intradermally, as above: (*E*) with 50 μl of 0.05 mM xanthine containing 0.004 unit of xanthine oxidase per ml, added immediately before injection; or (*F*) in addition, 19 μg of superoxide dismutase along with the superoxide generating system. Animals were sacrificed 3 hr later, and the sites were examined as above. The generation of superoxide *in situ* resulted in the diffuse infiltrate seen in *E*.

The possibility that superoxide mediates the neutrophil infiltration by reacting with plasma components to generate activity chemotactic for neutrophils was investigated. Human plasma was exposed *in vitro* to superoxide generated by the enzymatic oxidation of xanthine by xanthine oxidase. The chemotactic activity of the resulting solutions was assessed by the Boyden chamber technique (Table 2). Catalase or superoxide dismutase was added along with the superoxide-generating system to assess the participation of hydrogen peroxide or superoxide, respectively, in the activation of chemotactic activity. For each experiment, individual positive control (10% superoxide-treated plasma) and negative control (10% untreated plasma) values are listed because day-to-day and donor variability precluded direct comparison of values obtained for various experiments. Exposure of plasma to xanthine and xanthine oxidase resulted in generation of chemotactic activity for neutrophils. Superoxide dismutase abolished the chemotactic response when added before, but not after, exposure of

Table 2. Characterization of superoxide-dependent chemotactic activity by Boyden technique

Exp.	Description*	Cells/field†	% inhibition
1	Normal plasma	4.3 ± 3.0	—
	Superoxide-treated plasma	16.2 ± 4.2	—
	No xanthine	6.4 ± 2.5	87
	No xanthine oxidase	4.3 ± 1.6	100
2	Normal plasma	1.3 ± 0.9	—
	Superoxide-treated plasma	20.7 ± 3.9	—
	Plus SOD at t = 0	1.5 ± 1.4	99
	Plus SOD at t = 60	19.0 ± 3.5	9
3	Normal plasma	2.3 ± 1.4	—
	Superoxide-treated plasma	16.3 ± 3.4	—
	Plus catalase at t = 0	14.2 ± 3.8	15
	Plus catalase at t = 60	12.3 ± 5.5	28
4	Normal plasma	20.0 ± 7.7	—
	Superoxide-treated plasma	90.3 ± 9.6	—
	Heated to 60° for 30 min	33.2 ± 5.3	81

Plasma was used at a concentration of 10% in Hanks' balanced salt solution. Superoxide-treated plasma was incubated for 1 hr at 37°C with 0.3 mM xanthine and 0.01 unit of xanthine oxidase per ml.
* Superoxide dismutase (SOD) was added at a concentration of 50 μg/ml at 0 min or after 60 min. Catalase was added at 83 μg/ml.
† Mean ± SEM.

plasma to superoxide. Catalase caused no significant reduction of chemotactic activity when added prior to xanthine oxidase. Heating superoxide-treated plasma for 30 min at 56 or 60°C resulted in a 53 or 81% reduction in activity, respectively. The activity stable to storage at 4°C for 24 hr was nondialyzable and was stable to lyophilization.

Fig. 2 illustrates the response of neutrophils to various concentrations of plasma activated with superoxide or to untreated plasma. The addition of superoxide dismutase to the plasma prior to superoxide exposure reduced the chemotactic activity by 50% at 10 μg/ml; 25 μg/ml totally prevented the production of the factor.

In order to determine whether superoxide-treated plasma was chemotactic or merely enhanced the random motility of the cells (chemokinesis), experiments were performed in Boyden chambers in which the gradient was either reversed (by placing the treated plasma in the upper chamber with the cells) or abolished (by placing treated plasma in both upper and lower chambers). A positive migration of cells was seen only in the presence of a positive gradient of superoxide-treated plasma. No enhancement of migration was seen with a negative gradient or when the factor was present in both chambers.

The possibility that the superoxide-dependent plasma factor might stimulate superoxide production by leukocytes was examined. Neutrophils were suspended at 2×10^7 cells/ml in Hanks' balanced salt solution with 10% superoxide-activated or normal plasma and 30 μM ferricytochrome *c*. Aliquots were taken at 10 and 20 min and centrifuged, and the absorbance at 550 nm was measured to assess the rate of cytochrome reduction. No stimulation of superoxide production was evident. Normal stimulation of superoxide was seen when the neutrophils were incubated with opsonized zymosan.

The possibility that the factor might induce degranulation was examined by monitoring release of acid phosphatase from neutrophils incubated in superoxide-activated plasma. In a volume of 1 ml, 2×10^6 neutrophils were incubated at 37°C in Hanks' balanced salt solution containing 10% plasma, superoxide-activated or normal. Zymosan particles suspended in 10% normal plasma served as the positive control. After 2 hr, the mixtures were centrifuged and the supernatants were assayed for acid phosphatase. Cells incubated in superoxide-

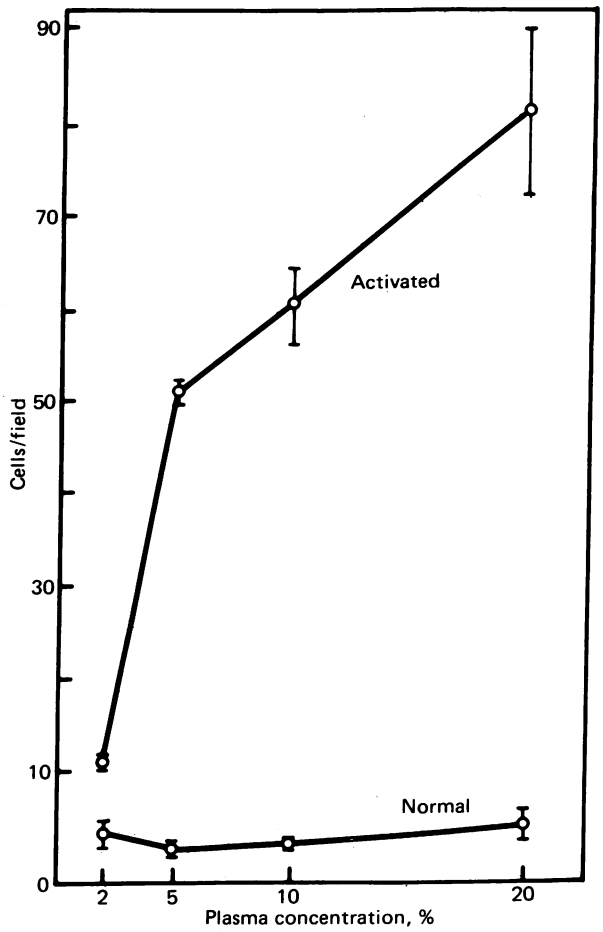

FIG. 2. Chemotactic response of neutrophils versus concentration of superoxide-treated plasma *in vitro*. For each concentration tested, the response to superoxide-treated plasma (activated) was markedly greater than the response to untreated plasma (normal). For superoxide exposure, plasma, at the concentrations indicated, was incubated with xanthine (0.3 mM) and xanthine oxidase (0.01 unit/ml) for 30 min at 30°C. Resultant solutions were assayed for chemotactic activity by the Boyden chamber method. Results are shown as mean ± SEM.

treated plasma showed no enhanced release of acid phosphatase compared to those incubated in untreated plasma. Zymosan-stimulated cells, however, released acid phosphatase as expected.

The ability of superoxide-treated plasma to effect neutrophil migration *in vivo* was assessed histologically after intradermal injection in rats. Both human and rat plasma exposed to superoxide elicited a marked infiltration of neutrophils, evident as early as 30 min after injection and increasing with time. Heavy infiltration was present 5 hr after injection (Fig. 1*C*). Control plasma, exposed to the superoxide-generating system after depletion of the substrate, elicited no marked leukocyte infiltration (Fig. 1*D*), indicating that generation of chemotactic activity was not due to xanthine oxidase *per se* or to the stable products of the superoxide-generating system, urate and hydrogen peroxide. No erythema, edema, or other manifestation of inflammation was evident at sites injected with chemotactically active, superoxide-treated plasma. Results identical to those obtained with human plasma were observed with rat plasma.

Rats were injected intradermally with xanthine and xanthine oxidase to assess the leukocyte response at sites of superoxide generation *in vivo* (Fig. 1*E*). A substantial infiltration of neutrophils was observed at injection sites although the response

was less than with superoxide-treated plasma. That the response was not due to xanthine oxidase or the stable products of xanthine oxidation was confirmed by injecting superoxide dismutase along with the superoxide-generating system. This resulted in no leukocyte infiltration (Fig. 1*F*). Like superoxide-treated plasma, the superoxide-generating system did not cause grossly observable edema or erythema.

Sephadex G-100 gel filtration of normal plasma resolved two major protein peaks; weak chemotactic activity was present only in the first (high molecular weight) peak (Fig. 3), as others have described (20). Activity in superoxide-treated plasma, however, appeared both in the high molecular weight peak and in the second protein peak, which was serum albumin. Chemotactically active fractions of the second peak were pooled, diluted, and applied to a Whatman DE-52 anion exchange column equilibrated with 0.1 M NaCl/5 mM phosphate, pH 7.2. Elution of the column with a continuous NaCl gradient (0.01–0.1 M) yielded four distinct protein peaks. Fractions of each peak were pooled, dialyzed, and assayed (without concentration) for chemotactic activity. Chemotactic activity was associated only with one protein peak, the major and last peak eluted. Polyacrylamide gel electrophoresis of the active material revealed a single protein which migrated to a position identical to that of commercial serum albumin.

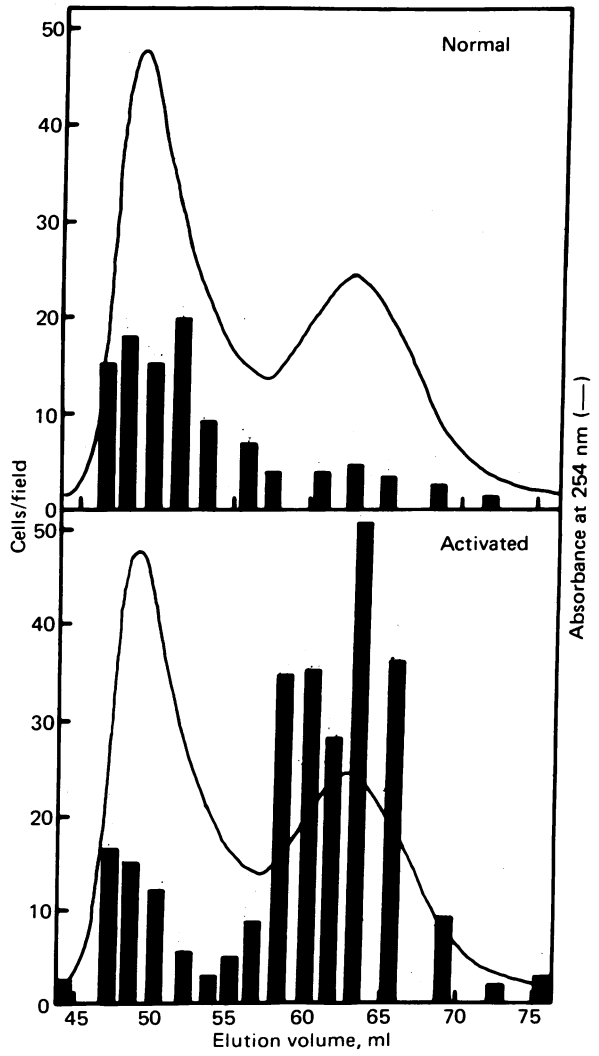

FIG. 3. Gel filtration chromatography of normal and activated plasma. Aliquots (5 ml) of normal or superoxide-activated human plasma (30%) were applied to a column of Sephadex G-100 (1.5 × 84 cm) equilibrated with 0.15 M NaCl. Activated plasma was prepared as in Fig. 2. Protein was monitored at 254 nm. Fractions were assayed for chemotactic activity by the Boyden technique.

Albumin so purified from untreated human plasma became weakly chemotactic when exposed to superoxide. Neither commercially obtained bovine serum albumin nor fatty acid-free human serum albumin showed enhanced chemotactic activity after superoxide exposure.

We speculate that the precursor of activity in plasma was a fatty acid or other lipid bound to serum albumin. To test this, lipids were extracted from untreated plasma with chloroform. The dried extract was insoluble in aqueous solutions, but it dissolved in dimethyl sulfoxide. A suspension of the dimethyl sulfoxide extract in Hanks' balanced salt solution was opalescent and had no chemotactic activity for neutrophils, even after exposure to superoxide (Table 3). Clear solutions resulted when the dimethyl sulfoxide solution of extract was mixed with solutions of ovalbumin or serum albumin. The ovalbumin-solubilized extract had no chemotactic activity. Albumin-solubilized extracts, however, did display activity chemotactic for neutrophils in comparison to albumin solutions alone. More importantly, this activity was substantially increased when the albumin-extract solutions were exposed to superoxide. No activity resulted when the ovalbumin-solubilized extracts were exposed to superoxide. Finally, superoxide dismutase inhibited the chemotactic activation of the albumin-solubilized extracts upon exposure to superoxide.

Oxidized lipids of platelet origin are chemotactic for neutrophils and eosinophils (21). To determine whether platelets might be the source of the superoxide-dependent chemotactic factor, we compared the chemotactic potential of platelet-rich plasma with that of plasma rendered platelet-poor by high-speed centrifugation. Upon exposure to superoxide, equivalent amounts of chemotactic activity were generated, indicating no dependence on the presence of platelets.

DISCUSSION

Superoxide reacts with a precursor in plasma to generate a factor chemotactic for neutrophils. The factor has not been identified. That its generation is dependent on superoxide *per se* is supported by experiments demonstrating that superoxide dismutase, but not catalase, inhibits its generation. Thus, the activity does not require the generation of hydroxyl radical or other secondary radicals. The factor and its precursor have ion exchange, electrophoretic, and size properties identical to those of serum albumin. Fatty acid-free human serum albumin could not be rendered chemotactic by exposure to superoxide. However, when a lipid extract of untreated plasma was dissolved in fatty acid-free albumin, the resulting material could be chemotactically activated by superoxide. The affinity of albumin for lipids in plasma is well known (22). That this specific interaction may be critical in expression of chemotactic activity by lipids is supported by the lack of superoxide-dependent activity in lipid extracts dissolved in Hanks' balanced salt solution or ovalbumin.

Table 3. Reconstitution of superoxide-dependent chemotactic activity assessed by Boyden technique

Test solution*	Cells/field
Extract in serum albumin	15.6
+ superoxide-generating system	54.8
+ superoxide-generating system + SOD†	14.6
Extract in ovalbumin	3.5
+ superoxide-generating system	2.0
Albumin alone	1.2
+ superoxide-generating system	2.6

* Chloroform extract of plasma was prepared and dissolved in 0.2% fatty acid-free human serum albumin or ovalbumin at pH 7.2. The superoxide-generating system is described in Table 2.

† Superoxide dismutase (SOD) was added at 50 μg/ml, prior to the addition of xanthine oxidase.

Superoxide is unreactive toward purified polyunsaturated fatty acids. However, it can reduce hydroperoxides, presumably to alkoxy radicals (RO·) which may abstract hydrogen to become hydroxy fatty acids (23). Various lipids have previously been demonstrated to have chemotactic activity for neutrophils (21, 24–28). Several of these are derived from arachidonic acid (21, 27, 28). The arachidonic acid oxidation product hydroperoxyicosatetraenoic acid (HPETE) is chemotactic, but the reduction product of this hydroperoxide, hydroxyicosatetraenoic acid (HETE) is more potently chemotactic (27). Perez and Goldstein (28) demonstrated that incubation *in vitro* of purified arachidonic acid with a superoxide-generating system results in the formation of a factor with potent chemotactic activity. The generation of this activity was inhibited by scavengers of O_2^-·, H_2O_2, OH·, or singlet oxygen. These data support a Haber–Weiss mechanism and indicate that superoxide is not directly responsible for the production of the chemotactic activity, unlike the results we report here. It is possible that the two chemotactic factors are structurally analogous or even identical but derived by different mechanisms: theirs by the direct radical-mediated hydroxylation of a fatty acid, and ours by the superoxide-dependent reduction of a pre-existing hydroperoxy fatty acid.

That the superoxide-dependent chemotactic activity we describe participates in the inflammatory process is suggested by the ability of superoxide-activated plasma or of a superoxide-generating system to attract leukocytes *in vivo* and by the ability of superoxide dismutase derivatives to inhibit leukocyte migration to sites of inflammatory challenge. Superoxide dismutase does not inhibit chemotaxis directly. During inflammation, neutrophils produce and release superoxide, a property presumably acquired as an antimicrobial mechanism. We propose that the superoxide produced by stimulated neutrophils reacts with the plasma precursor to produce chemotactic activity. Neutrophils may have been drawn to the site initially by complement or bacterial or other chemotactic factors. The superoxide-dependent activity perpetuates and amplifies the arrival of inflammatory cells by continuously providing a neutrophil chemo-attractant for the duration of superoxide production. Upon elimination of the inflammatory stimulus, superoxide production would subside and thus preclude further generation of chemotactic activity, allowing the situation to resolve and return to normalcy.

The anti-inflammatory activity of superoxide dismutase has been seen in several animal models of induced inflammation as well as in clinical trials with humans (1, 2, 13, 29). The assumed action of the enzyme has been the protection of tissues from direct cytotoxicity of neutrophil-generated superoxide. By this mechanism, other cytotoxic, neutrophil-mediated events (such as release of myeloperoxidase and hydrogen peroxide, neutral proteases, and other hydrolytic granular enzymes) should be unchecked by the presence of superoxide dismutase. Thus, the enzyme's anti-inflammatory activity should reflect only that fraction of the total cytotoxicity due to superoxide production. From data contained in this report, we now believe that the enzyme's anti-inflammatory action is the prevention of the formation of superoxide-dependent chemotactic activity. This action prevents the accumulation of inflammatory cells at the site of a potential lesion, thereby preventing all neutrophil-mediated cytotoxic events. Although many chemotactic factors exist and may play roles at various stages in the development of inflammation, we suggest that the superoxide-dependent factor is critically important in the models we have examined. Its role may be that of the quantitatively dominant chemotactic factor, or it may play an early role whereby its generation is necessary for the subsequent generation or release of the quantitatively dominant factor(s).

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