PRIMING OF MACROPHAGES FOR ENHANCED OXIDATIVE METABOLISM BY EXPOSURE TO PROTEOLYTIC ENZYMES

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Enhanced spreading of macrophages on glass or plastic surfaces has been defined as a property of activated macrophages (1, 2). Rabinovitch and DeStefano (3) demonstrated that incubation for 30 min with trypsin, pronase, papain, or subtilisin induced marked spreading in normal mouse peritoneal macrophages attached to glass; and Götzé et al. (4) reported that activated factor B of the properdin system (Bb), when active as a protease, stimulated pronounced macrophage spreading within 60 min. Increased activity of the hexose monophosphate shunt in resting (nonphagocytic) macrophages also was noted after exposure to proteolytic enzymes (2).

Macrophages activated by infection or elicited by injection of inflammatory agents are primed so that they undergo an enhanced oxidative metabolic response when stimulated by phagocytosis or contact with surface-active agents such as phorbol myristate acetate (PMA; 5, 6). We report here that brief exposure to proteinases in vitro also primes macrophages for greater oxidative metabolism, measured as stimulated release of superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$).

Materials and Methods

Macrophages. Peritoneal macrophages were harvested from mice as previously described (6). Washed cells were suspended in medium supplemented with 20% heat-inactivated fetal calf serum (FCS; 6), and 3–4 x 10$^6$ cells in a total volume of 1 ml were plated on 35-mm tissue culture dishes. After incubation for 120 min at 37°C in 5% CO$_2$-95% air, plated cells were washed vigorously twice with Hanks' balanced salt solution (HBSS; Grand Island Biological Co., Grand Island, N. Y.), then cultured overnight in medium with 20% FCS, penicillin, and streptomycin (6). The cells adherent after overnight culture, as estimated by differential counts of stained smears (6) and by phagocytic capacity for candida (7), were >97% macrophages and <1% granulocytes. The protein content of the dishes at the time of assay was 40–90 µg/dish. Thioglycollate-elicited cells were obtained from the peritoneum 4 d after intraperitoneal injection of thioglycollate medium (6).

Treatment of Macrophages with Proteolytic Enzymes. After overnight culture, macrophages were washed twice with HBSS then incubated with trypsin (Worthington Biochemical Corp., Freehold, N. J.), pronase (protease from Streptomyces griseus, type VI; Sigma Chemical Co., St. Louis, Mo.), chymotrypsin (Worthington Biochemical Corp.), or papain (Sigma Chemical Co.). The papain was "activated" immediately before use by incubation at 2.5 mg/ml with 2.5 mM dithiothreitol (P-L Biochemicals, Inc., Milwaukee, Wis.) for 10 min at 37°C in 0.1 M sodium phosphate buffer, pH 7.2, with 0.01 M EDTA. The enzymes in HBSS were added to the macrophages at final concentrations of 1–250 µg/ml.

Macrophages on control dishes were incubated with HBSS alone or with HBSS containing...
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FCS, bovine serum albumin (BSA; Sigma Chemical Co.), lysozyme (grade I, Sigma Chemical Co.), or enzymes that had been pretreated with diisopropyl fluorophosphate (DFP; Sigma Chemical Co.) or L-l-tosylamide-2-phenylethyl-chloromethyl ketone (TPCK, Sigma Chemical Co.). All control proteins were used in concentrations equivalent to those of the proteolytic enzyme under study. DFP inactivation of trypsin, pronase, and papain, and TPCK inactivation of chymotrypsin were accomplished by incubation of the enzyme with a threefold molar excess of DFP or TPCK for 60 min at 23°C; the mixture was dialyzed overnight against HBSS before use. Trypsin, pronase, and papain, before and after inactivation, were tested for their capacity to hydrolyze \( \alpha-N \)-benzoyl-L-arginine ethyl ester (Sigma Chemical Co.), and chymotrypsin was tested for its ability to cleave \( N \)-benzoyl-L-tyrosine ethyl ester HCl (Sigma Chemical Co.), as described in the Worthington enzyme manual (Worthington Biochemical Corp.).

After incubation with the enzymes or control preparations for 10-30 min at 37°C, the fluid was removed by aspiration, and the cells were incubated for 5 min in 1 ml HBSS with 20% heat-inactivated FCS. After two washes in HBSS, the cells were assayed for their capacity to generate \( \mathrm{O}_2^- \) or \( \mathrm{H}_2\mathrm{O}_2 \), as described below. Preincubation for 30 min with any of the four enzymes at a concentration of 500 \( \mu \)g/ml did not affect viability of the macrophages (trypan blue dye exclusion) or the capacity to ingest sheep erythrocytes coated with IgG antibody (8).

Treatment of Macrophages with Neuraminidase. After overnight culture, macrophages were washed twice with Dulbecco's phosphate-buffered saline with calcium and magnesium ions (DPBS; Grand Island Biological Co.), then incubated for 20 min at 37°C with neuraminidase (type V or type IX, both from \textit{Candida perfringens}; Sigma Chemical Co.), 0.06 U in 1 ml DPBS. The reaction was stopped by removal of the supernates, and the macrophages were washed twice with DPBS, then tested for their capacity to release \( \mathrm{O}_2^- \). Control dishes were incubated with DPBS alone. Activity of the neuraminidase was quantitated before use by its capacity to release sialic acid from mucin (type I; Sigma Chemical Co.), using the method described in the Worthington enzyme manual.

After the \( \mathrm{O}_2^- \) assay had been completed, the cells were washed twice with DPBS, then 1 ml of 0.1 N \( \mathrm{H}_2\mathrm{SO}_4 \) was added to the dish and the cells were scraped off with a rubber policeman. Digested cells from five neuraminidase-treated and five control plates were each pooled. Supernates from buffer or neuraminidase pretreatment were also adjusted to 0.1 N \( \mathrm{H}_2\mathrm{SO}_4 \). The four pools were hydrolyzed at 80°C for 1 h, then dried by rotary evaporation. Each of the four dried samples was then redissolved in 0.5 ml water, and the sialic acid content was determined by the procedure of Warren (9), with correction for interfering substances. Absorbance of the supernate samples was corrected for the absorbance of DPBS or DPBS-neuraminidase.

Release of \( \mathrm{O}_2^- \) and \( \mathrm{H}_2\mathrm{O}_2 \). The release of \( \mathrm{O}_2^- \) was quantitated as previously described (6). The reaction was started by addition of ferricytochrome \( c \) and either PMA (Consolidated Midland Corp., Brewster, N. Y.), 100-500 ng/ml (6), \textit{Candida parapsilosis} in HBSS at an organism to macrophage ratio of 1:1-3:1 (7), or opsonized zymosan, 1 mg/ml (6). Each reaction was run in duplicate or triplicate. Controls contained the same reaction mixture without macrophages. Incubation was at 37°C for 90 min with PMA or 60 min with \textit{C. parapsilosis} or zymosan. The extent of \( \mathrm{O}_2^- \) release was expressed in relationship to the protein content of each culture dish (6). In initial experiments, the protein content was measured for three to five dishes that were only preincubated with enzyme and washed; the values obtained were equivalent to those obtained after the \( \mathrm{O}_2^- \) assay.

The release of \( \mathrm{H}_2\mathrm{O}_2 \) by macrophages was quantitated by a modification of the technique of Nathan and Root (5). The reaction was started by addition to washed cells of a mixture of scopoletin (Sigma Chemical Co.), 3.3 \( \mu \)M, horseradish peroxidase (type II; Sigma Chemical Co.), 0.02 mg/ml, and PMA, 0.5 \( \mu \)g/ml, all in Krebs-Ringer phosphate buffer with 2 mg/ml dextrose, to a volume of 1.5 ml. Incubation was for 10 min. The reaction mixture was removed, cleared by centrifugation, and diluted with buffer; the fluorescence of scopoletin was determined (3), using scopoletin in buffer to obtain the 100% value. That the decrease in scopoletin fluorescence in this system was dependent upon oxidation by \( \mathrm{H}_2\mathrm{O}_2 \) was demonstrated by elimination of 85-93% of the decrease by addition of catalase, 4,000 U/ml (Sigma Chemical Co.; 7), and elimination of all fluorescence change in the absence of peroxidase. Addition to the reaction mixture of 30 mg/ml superoxide dismutase, which catalyzes the conversion of \( \mathrm{O}_2^- \) to \( \mathrm{H}_2\mathrm{O}_2 \), decreased slightly the extent of scopoletin oxidation; mannitol, 50 mM, and benzoate, 20 mM, which scavenge hydroxyl radical (7), had no effect.
Table I
Effect of Preincubation with Proteolytic Enzymes on Release of O_2^- and \(\text{H}_2\text{O}_2\) by Resident Mouse Peritoneal Macrophages

| Enzyme* | Release of O_2^- \(\mu\)mol/mg | Release of \(\text{H}_2\text{O}_2\) \(\mu\)mol/mg |
|---------|-------------------------------|---------------------------------|
|         | Stimulus                      | Stimulus                       |
| None    | 0 (3)                         | 54 ± 17 (7)                    |
| Trypsin | 0 (3)                         | 255 ± 23 (13)                  |
| Pronase | 0 (3)                         | 303 ± 36 (8)                   |
| Chymotrypsin | 0 (4)               | 414 ± 41 (4)                   |
| Papain  | 365 ± 34 (3)                  | 385 ± 34 (3)                   |

* Macrophages were preincubated for 10 min with a control preparation or with enzyme, 10–250 \(\mu\)g/ml.

Results

Macrophages preincubated for 10 min with trypsin, pronase, chymotrypsin, or papain, and then washed were primed to release two to six times more \(\text{O}_2^-\) when stimulated by PMA or viable \(C. \text{parapsilosis}\) (Table I). PMA-stimulated release of \(\text{H}_2\text{O}_2\) was similarly enhanced. Enzyme pretreatment did not in itself stimulate oxidative metabolism in that there was no \(\text{O}_2^-\) release from trypsin- or pronase-treated macrophages in the absence of PMA or candida (Table I). Preincubation of macrophages with enzymes inactivated by pretreatment with DFP or TPCK (see Materials and Methods; two experiments with each enzyme), or preincubation with FCS, BSA, or boiled lysozyme (four to six experiments with each) resulted in PMA-stimulated \(\text{O}_2^-\) release equivalent to that obtained by preincubation with HBSS alone. In two experiments, the release of \(\text{O}_2^-\) from PMA-stimulated thioglycollate-elicited macrophages, which are already fully primed (6), was 474 \(\mu\)mol/mg (mean); this high release was not modified by pretreatment with trypsin or pronase (means of 460 and 425 \(\mu\)mol/mg, respectively).

Because of possible contamination of enzyme preparations with bacterial lipopolysaccharide (LPS), which can prime macrophages for greater \(\text{O}_2^-\) release (10), cells were preincubated with phenol-extracted LPS from \(E. \text{coli} \ K235\) (10) at concentrations of 0.01–10 \(\mu\)g/ml. There was no significant priming by LPS during 30 min of preincubation.

The extent of priming varied with the concentration of the enzyme present during preincubation (Fig. 1). In most experiments, a concentration of 50 \(\mu\)g/ml gave close to the maximal effect, little further priming being noted with concentrations of 100 \(\mu\)g/ml or 250 \(\mu\)g/ml. Preincubation for 10 min gave approximately the same effect as preincubation for 20 or 30 min (two or three experiments with each enzyme).

Treatment of human neutrophils with neuraminidase has been reported to decrease their capacity to release \(\text{O}_2^-\) on contact with latex particles or concanavalin A (11). Preincubation of normal mouse macrophages with neuraminidase, either type V, which is contaminated with some proteolytic activity, or type IX, more highly purified, did not decrease their capacity to release \(\text{O}_2^-\) when stimulated by PMA or opsonized zymosan (Table II). Similar results were obtained in two experiments using thioglycollate-elicited macrophages. In four of the experiments of Table II, pretreat-
**Fig. 1.** Release of \( \text{O}_2^- \) by resident mouse peritoneal macrophages preincubated for 10 min with varying concentrations of proteolytic enzyme, then stimulated with PMA. The extent of \( \text{O}_2^- \) release by enzyme-treated cells was compared in each experiment to \( \text{O}_2^- \) release by cells preincubated in a control preparation, and the ratio is plotted. Representative experiments are shown from six done with trypsin, five each done with pronase and papain, and four done with chymotrypsin, each with at least three concentrations of enzyme. The minimal concentration of enzyme that consistently primed cells for greater \( \text{O}_2^- \) release was \( \approx 1 \mu\text{g/ml} \) for trypsin, pronase, and papain, and \( 4 \mu\text{g/ml} \) for chymotrypsin. □, pronase; (△), papain; (■), chymotrypsin; (○), trypsin.

**Table II**

*Release of \( \text{O}_2^- \) by Macrophages Preincubated with Neuraminidase*

| Enzyme*          | PMA‡  | Opsonized zymosan‡ |
|------------------|-------|--------------------|
| None             | 146 ± 25 (7) | 302 ± 53 (3) |
| Neuraminidase    | 158 ± 30 (10) | 313 ± 51 (6) |

*Macrophages were preincubated for 20 min with buffer or enzyme, 0.06 U, in paired experiments (with PMA, six experiments with type IX and four with type V; with zymosan, three experiments with each enzyme).

‡Mean ± SEM. The number of experiments, performed in replicates of two to five, is given in parentheses. Results were equivalent with type V and type IX enzyme, with either stimulus.

Discussion

The results reported here indicate that exposure of cultured macrophages to proteolytic enzymes, although not in itself stimulatory of \( \text{O}_2^- \) release, primes the cells to release more \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) on membrane perturbation. The mechanism for this priming remains to be defined. Simply an increase in binding of the stimuli might explain this effect. However, the PMA concentration used was >50 times in excess of that required to give a peak response (D. A. Chadwick and R. B. Johnston, Jr., unpublished data), which makes this explanation unlikely, at least with PMA. Moreover, the effect was noted with two different types of stimuli.

Perhaps a more likely explanation for the priming effect of proteolytic enzymes
would be an alteration in the plasma membrane such that the stimuli interact more effectively with structures associated with triggering the respiratory burst. This possibility is in keeping with the demonstration that macrophages from inflammatory exudates, which are primed to produce markedly increased amounts of \( \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) when stimulated (5, 6), possess qualitative and quantitative differences, compared with resident cells, in exteriorly disposed plasma membrane polypeptides (12). Internalization and retention of enzymatically active trypsin has been shown with fibroblasts (13), however, and the potential of surface proteolysis to modulate intracellular, as well as membrane, events has been shown in work with various cell types (14–18). Incubation with trypsin has been reported to activate adenylate cyclase (14), and to induce secretion of plasminogen activator and collagenase in fibroblasts (15), to increase cyclic AMP levels in lymphocytes (16), and to activate the sodium pump in certain erythrocytes (17). Neutral proteases induced increased synthesis and release of arachidonic acid metabolites by mouse peritoneal macrophages (18). Analysis of the mechanism by which proteolysis primes the cell for a greater \( \text{O}_2^- \) response could lead to better understanding of how membrane perturbation initiates the respiratory burst, and, perhaps, how activation of macrophages permits an enhanced oxidative metabolic response.

The priming for enhanced oxidative metabolism achieved by proteolytic enzyme treatment was equivalent to that seen with infection-activated and inflammatory macrophages (5, 6). Thus, it appears that if the oxidase responsible for the respiratory burst in macrophages is on the outside of the cell, as has been suggested for neutrophils, it is not subject to inactivation by proteolysis.

Concentrations of lysosomal hydrolases are believed to be high in areas of inflammation (19), and neutral proteases are released during activation of the complement and clotting systems. The effect of proteolysis on macrophages described here raises the possibility that proteinases present at inflammatory sites could prime macrophages to undergo a greater oxidative metabolic response to contact with foreign material. Such priming has, in fact, been noted in macrophages obtained from inflammatory exudates (6). An enhanced oxidative response could, in turn, contribute to the increased killing of microorganisms by activated macrophages or to the tissue damage that results from inflammation.

**Summary**

Preincubation for 10–30 min with trypsin, pronase, chymotrypsin, or papain primed macrophages to undergo a twofold to sixfold increase in oxidative metabolism, measured as release of superoxide anion or hydrogen peroxide, during stimulation by phorbol myristate acetate or ingestion of *Candida parapsilosis*. Preincubation of macrophages with inactivated proteases, nonenzyme proteins, or neuraminidase did not affect their oxidative response. Exposure of macrophages to proteases generated at sites of inflammation could prime these cells for a more effective oxidative response to phagocytosis or for greater tissue damage from release of toxic oxygen metabolites.

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