

Evidence that Proteases are Involved in Superoxide Production by Human Polymorphonuclear Leukocytes and Monocytes

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ABSTRACT The possible participation of proteases in superoxide (O_2^-) production by human polymorphonuclear leukocytes (PMN) and monocytes was explored using various protease inhibitors and substrates. Protease inhibitors used included naturally occurring inhibitors of serine proteases and synthetic inhibitors that modify the active site of serine proteases. Substrates used were synthetic substrates of the chymotrypsin type as well as trypsin type of protease. All these inhibitors and substrates inhibited O_2^- production by human PMN and monocytes induced by cytochalasin E and concanavalin A, though PMN were more sensitive to these inhibitors and substrates than monocytes. Inhibition appeared rapidly even when the inhibitors were added at the same time as the stimulants, during the "induction time of O_2^- production" or at the time of maximum O_2^- production, whereas much greater inhibition was observed when the cells were preincubated with the inhibitors. These observations suggest that enzymatically active serine proteases are essential for these phagocytic cells to initiate and maintain the O_2^- production in response to the stimuli. The inhibitory effect of the inhibitor and substrate for chymotrypsin type protease was greater than that of those substances for trypsin-type protease. Macromolecular inhibitors also inhibited the O_2^- production. These findings suggest that the serine proteases involved in the O_2^- production by human PMN and monocytes are similar to chymotrypsin rather than trypsin, and are possibly located at the cell surface membrane.

INTRODUCTION

Potent inactivators of serine proteases (esterases) can inhibit many functions of phagocytic cells, in-

cluding chemotaxis, phagocytosis, degranulation, and superoxide (O_2^-) production (1-9). The available evidence suggests that active and stimulus-activated esterases are required for chemotaxis and phagocytosis (1-5). The inhibition profiles of chemotaxis of rabbit polymorphonuclear leukocytes (PMN)¹ given by the several phosphonate esters indicate that the esterases are similar to chymotrypsin rather than trypsin, although not completely similar to chymotrypsin (1). The chymotrypsin type proteases are also required for the phagocytosis of antigen-antibody complexes by guinea pig peritoneal macrophages (6). We have previously reported that chymotrypsin-like serine proteases are involved in the O_2^- production by human PMN and that they are located at the cell surface membrane (9). In this paper, we investigated further the characteristics of the serine proteases involved in the O_2^- production by human PMN and extended the study to human peripheral blood monocytes. For this purpose, we used irreversible serine protease inhibitors, phenylmethylsulfonyl fluoride (PMSF), L-1-tosylamido-2-phenylethyl-chloromethyl ketone (TPCK) and N- α -p-tosyl-L-lysine-chloromethyl ketone (TLCK); naturally occurring macromolecular inhibitors, aprotinin and soybean trypsin inhibitor (SBTI); and synthetic substrates for serine proteases, N-benzoyl-L-tyrosine ethyl ester (BTEE) and p-tosyl-L-arginine methyl ester (TAME). PMSF is an active site-serine sulfonylating agent, and inhibits chymotrypsin and trypsin irreversibly, although it is more reactive toward chymotrypsin (10). TPCK is an active-site histidine alkylating agent and a specific inhibitor of chymotrypsin (11, 12). Trypsin is not affected by TPCK. TLCK is an active-site histidine

¹Abbreviations used in this paper: BTEE, N-benzoyl-L-tyrosine ethyl ester; Con A, concanavalin A; Cyt E, cytochalasin E; PMN, polymorphonuclear leukocytes; PMSF, phenylmethyl-sulfonyl fluoride; SBTI, soybean trypsin inhibitor; TAME, p-tosyl-L-arginine methyl ester; TLCK, N- α -p-tosyl-L-lysine-chloromethyl ketone; TPCK, L-1-tosylamido-2-phenylethyl-chloromethyl ketone.

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alkylating agent and is a specific inhibitor of trypsin (11, 13). Chymotrypsin is not affected by TLCK. Inasmuch as serine proteases have serine and histidine residues in the active center, it would be expected that the possible participation of serine proteases in the O_2^- production by human PMN and monocytes could be appropriately explored using PMSF, TPCK, and TLCK. Aprotinin (14) and SBTI (15) are reversible inhibitors, and inhibit trypsin as well as chymotrypsin, although they are more reactive toward trypsin. The location of the serine proteases can be appropriately explored using these macromolecular inhibitors, which can hardly penetrate the cell surface membrane. BTEE and TAME are hydrolyzed preferentially by chymotrypsin and trypsin, respectively (16). It would be expected that synthetic substrates for serine proteases could impair the physiological function of the serine proteases by competing with natural substrates for the serine proteases.

METHODS

Reagents. Cytochalasin E (Cyt E) was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis.; concanavalin A (Con A) grade IV, cytochrome C type VI, superoxide dismutase, TPCK, TLCK, SBTI, PMSF, BTEE, and TAME from Sigma Chemical Co., St. Louis, Mo.; aprotinin² from Behring Institute, West Germany, Cyt E, TPCK, BTEE, and PMSF were dissolved in dimethylsulfoxide and diluted with HEPES-saline (isotonic saline solution buffered with 5 mM N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid, pH 7.4) immediately before use. The final concentration of dimethyl sulfoxide in the reaction mixture was 2.5–5 μ l/ml and the same concentration of dimethyl sulfoxide was added to the controls.

Preparation of cells. Heparinized venous blood from healthy adult donors was allowed to sediment at room temperature for 30 min after mixing with the same volume of 3% dextran in isotonic saline. Pure PMN and mononuclear cell fractions were obtained from the leukocyte-rich supernates by the Conray-Ficoll method (17) (Conray, Mallinckrodt Inc., St. Louis, Mo.; Ficoll, Pharmacia Fine Chemicals, Inc., Piscataway, N. J.). Mononuclear cell fraction was suspended in HEPES-saline, washed twice, and resuspended in the same buffer to be used as monocyte fraction, which contained 15–25% monocytes and <1% PMN by morphological criteria. The rest was the lymphocytes. Contaminated erythrocytes in PMN fraction were removed by hypotonic lysis. PMN fraction was suspended in HEPES-saline and contained more than 99% PMN. To obtain pure lymphocyte preparations, the mononuclear cells in McCoy's 5A (5×10^9 /ml) were preincubated with carbonyl iron particles for 30 min at 37°C, and phagocytic cells were eliminated by the magnet. The nonphagocytic mononuclear cell preparations obtained by this procedure contained more than 99% lymphocytes by morphological criteria. The lymphocyte preparations were washed and suspended in HEPES-saline.

Determination of PMN and monocyte O_2^- production. O_2^- was assayed by the reduction of ferricytochrome C, spec-

trophotometrically, and the continuous assay was performed in a Hitachi 557 spectrophotometer (a double wave-length spectrophotometer with end-on photomultiplier; Hitachi Ltd., Tokyo), equipped with thermostated cuvette holder as described (9). The cell suspension was added to a 1-ml cuvette containing 2 mM glucose, 66 μ M ferricytochrome C and 1 mM $CaCl_2$ with or without test materials to obtain final volume of 0.99 ml. Final cell concentration was $2-4 \times 10^5$ PMN/ml or $1.3-2 \times 10^6$ mononuclear cells/ml. The reaction mixture in a cuvette was preincubated at 37°C for 3 min for protease inhibitors and 10 min for synthetic substrates for serine proteases, respectively. The cuvette was put in a thermostated cuvette holder (37°C) of a spectrophotometer and the reduction of cytochrome C was measured at 550 nm with a reference wave length at 540 nm. Cyt E (5 μ l; final concentration, 5 μ g/ml) and Con A (5 μ l; final concentration, 50 μ g/ml) were added simultaneously to the reaction mixture in a cuvette using a plastic rod, while the time-course of cytochrome C reduction was followed on the recorder. Cytochrome C reduction by human PMN and monocytes stimulated by Cyt E and Con A was completely abolished by superoxide dismutase (20 μ g/ml) and suggested to be specific for O_2^- as described (18). Results with three or four experiments were averaged and converted to nanomoles of cytochrome C reduced by using $E_{550-540nm}$ (ferrocyclochrome C minus ferricytochrome C) = $15.5 \times 10^3 M^{-1} cm^{-1}$ (18). Although the cytochrome C reduction was not actually linear with time, we used for the assay an apparently linear portion, and the rate of cytochrome C reduction in the resting state was subtracted from that in the stimulated state. In these studies, cell viability by erythrosine B dye exclusion test was always checked after the assay of O_2^- production, and was >95% even after the treatment with various compounds.

RESULTS

Characteristics of the O_2^- production by human PMN and monocytes induced by Cyt E and Con A. Not only human PMN (18–22) but also human monocytes were able to release O_2^- in response to Cyt E or Con A, and marked enhancement of the O_2^- production was observed when Cyt E and Con A were added simultaneously (Fig. 1). However, the responsive patterns of monocytes to Cyt E and/or Con A were different from those of PMN. Cyt E was a more effective stimulant than Con A in the PMN O_2^- production, whereas Con A was a more effective stimulant than Cyt E in the monocyte O_2^- production. Furthermore, Con A-induced O_2^- production by PMN was continuous and linear, whereas that by monocytes was transient and ceased within 4–5 min. The O_2^- production by human PMN and monocytes was dependent on the concentration of Cyt E and Con A (Fig. 2) and the number of cells in the reaction mixture (data not shown). When phagocytic cells ingesting carbonyl iron particles were eliminated from the mononuclear cell fraction, Cyt E- and Con A-induced O_2^- production by the remaining cells (>99% lymphocytes) was negligible (data not shown), indicating that the O_2^- production by mononuclear cells is attributed to monocytes. In the following experiments, we used 5 μ g/ml Cyt E and 50 μ g/ml Con A simultaneously to induce the O_2^- production by human PMN and monocytes.

² Although we used aprotinin from Bayer Co. in the previous report (9), we used aprotinin from Behring Institute in the present experiments, because the inhibitory effect of the former was greater than that of the latter possibly because of the contaminated materials that were suspected spectrophotometrically by the absorbance spectra.

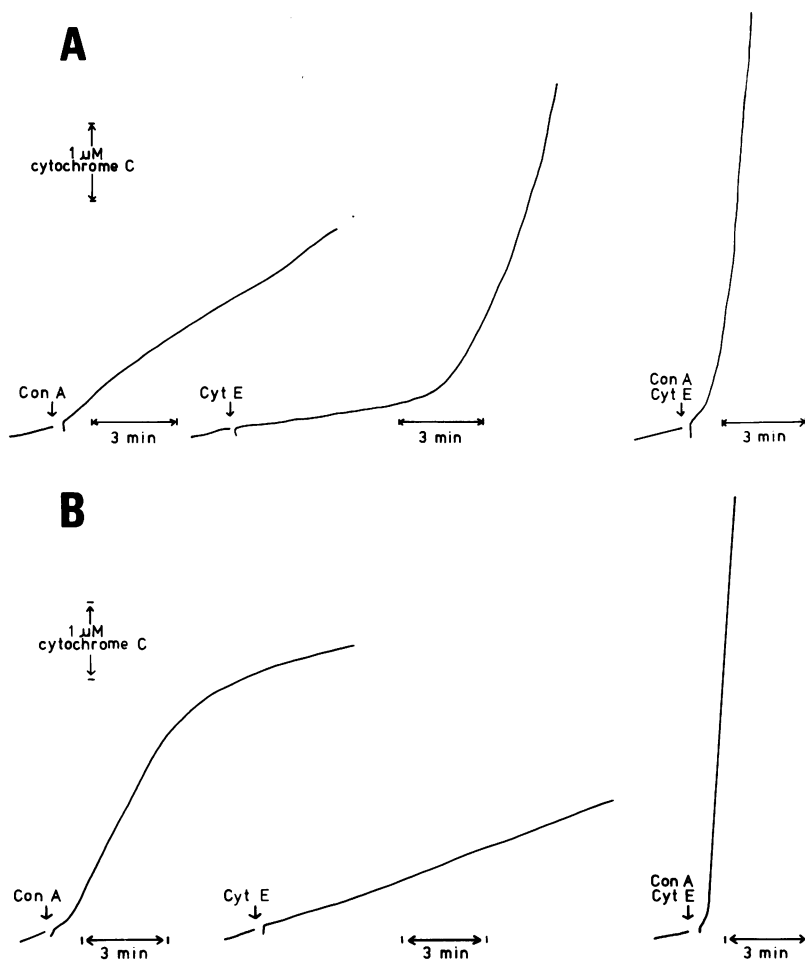


FIGURE 1 O_2^- production by human PMN (A) and monocytes (B) stimulated by Con A and/or Cyt E. PMN, $5 \times 10^6/\text{ml}$, and mononuclear cells, $1.2 \times 10^6/\text{ml}$ (monocytes 25%), were used. Left: Con A ($50 \mu\text{g}/\text{ml}$) alone was added. Median: Cyt E ($5 \mu\text{g}/\text{ml}$) alone was added. Right: Con A ($50 \mu\text{g}/\text{ml}$) and Cyt E ($5 \mu\text{g}/\text{ml}$) were added simultaneously.

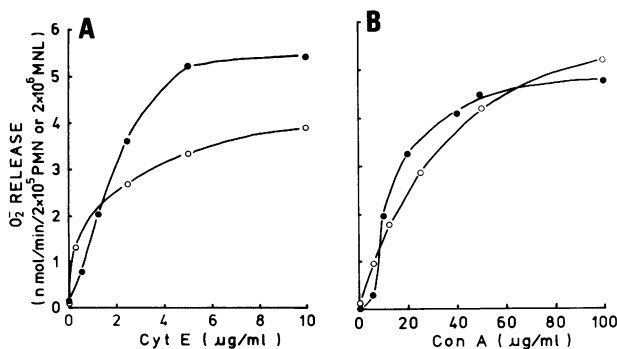


FIGURE 2 Enhancing effect of Cyt E (Con A) on the O_2^- production by human PMN and monocytes stimulated by Con A (Cyt E). PMN, $2 \times 10^5/\text{ml}$, and mononuclear cells (MNL), $2 \times 10^5/\text{ml}$, were used. (A) Con A ($50 \mu\text{g}/\text{ml}$) and indicated concentration of Cyt E were added simultaneously. (B) Cyt E ($5 \mu\text{g}/\text{ml}$) and indicated concentration of Con A were added simultaneously. \circ , PMN; \bullet , MNL.

Inhibition of PMN and monocyte O_2^- production by protease inhibitors. Cell suspensions in cuvettes were preincubated with various concentrations of protease inhibitors for 3 min at 37°C , before Cyt E ($5 \mu\text{g}/\text{ml}$) and Con A ($50 \mu\text{g}/\text{ml}$) were added. The O_2^- production by human PMN and monocytes was inhibited by various protease inhibitors in a dose-dependent fashion (Fig. 3) (9). The relative potencies of inhibitory effect were TPCK > TLCK > aprotinin > SBTI > PMSF on a molar basis for the PMN O_2^- production, and TPCK > aprotinin > TLCK = SBTI > PMSF for the monocyte O_2^- production. Typical results obtained with TPCK are shown in Fig. 4.

Inhibition appeared rapidly even when TPCK was added at the same time as the stimulants or added during the induction time of O_2^- production, whereas much greater inhibition was observed when the cells were preincubated with TPCK (Fig. 4C). And the

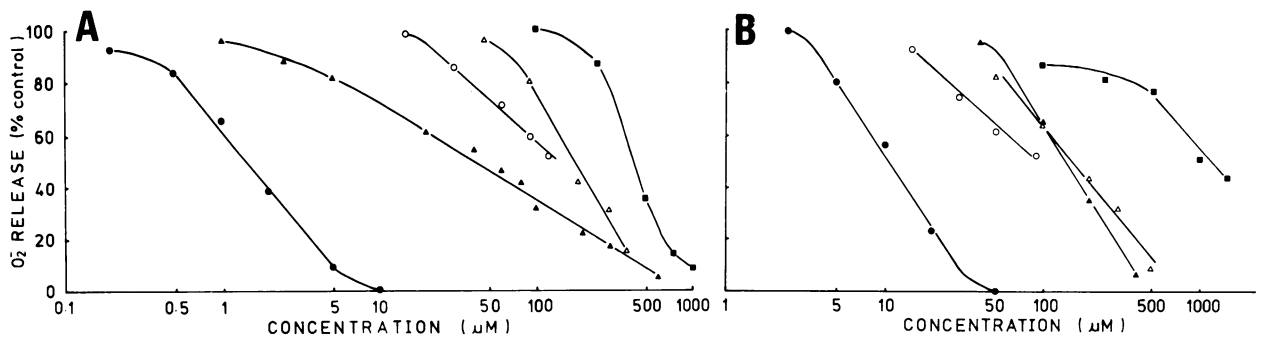


FIGURE 3 The inhibitory effect of various protease inhibitors on the O_2^- production by human PMN (A) and monocytes (B). Cell suspensions were preincubated with protease inhibitors for 3 min at $37^\circ C$ before Cyt E ($5 \mu g/ml$) and Con A ($50 \mu g/ml$) were added. ●, TPCK; ○, aprotinin; ▲, TLCK; △, SBTI; ■, PMSF.

inhibitory effect became greater as the preincubation time was prolonged (Fig. 5). Furthermore, TPCK was still able to inhibit the O_2^- production when added at the time of maximum O_2^- production (Fig. 4D) (9). The almost similar patterns of the inhibitory effect were observed when the other protease inhibitors were used. Compared to the inhibitory effect on the O_2^- production by human PMN, several times higher concentrations of protease inhibitors were required to obtain the same inhibition of the monocyte O_2^- production (Fig. 3).

Because the monocyte O_2^- production studies were done with the mononuclear cell fraction-containing lymphocytes, further control experiments were performed to exclude the possibility that lymphocytes might interfere with the inhibition by the protease inhibitors. This could be a result of a nonspecific protein effect or a result of enzyme activity in lymphocytes. To test this possibility, the effect of lymphocyte contamination on the inhibition of PMN O_2^- production was examined. No significant difference of inhibition by TPCK was seen between pure PMN preparation ($4 \times 10^5/ml$) and the mixture of pure PMN and pure lymphocytes (PMN $4 \times 10^5/ml$ and lymphocytes $1.2 \times 10^6/ml$) (data not shown). These observations indicate that lymphocytes in our monocyte fraction did not interfere with the inhibition by protease inhibitors.

Final cell concentration in the reaction mixture was $2-4 \times 10^5/ml$ for PMN and $1.3-2.0 \times 10^6/ml$ for mononuclear cells. To exclude the possibility that the large cell number of mononuclear cells might interfere with the inhibition by protease inhibitors, we investigated the inhibitory effect by protease inhibitors on the O_2^- production by PMN in the range of from $2 \times 10^5/ml$ to $2 \times 10^6/ml$, and no significant difference of inhibition profile was seen (data not shown).

Inhibition of PMN and monocyte O_2^- production by synthetic substrates for serine proteases. Cell sus-

pensions in cuvettes were preincubated with various concentrations of synthetic substrates for serine proteases for 10 min at $37^\circ C$, before Cyt E and Con A were added. The O_2^- production by human PMN and monocytes was inhibited in a dose-dependent fashion by synthetic substrates for serine proteases, including BTEE (substrate for chymotrypsin-type protease) and TAME (substrate for trypsin-type protease) (Fig. 6) (9, 16, 23, 24). BTEE was much more effective than TAME. As seen in the inhibition by protease inhibitors, PMN were also more sensitive to the synthetic substrates than monocytes.

Restoration of PMN and monocyte O_2^- production by removal of protease inhibitors. A remarkable restoration of PMN O_2^- production was seen when PMSF was removed from the milieu by a simple washing procedure after the preincubation for 5 min at $37^\circ C$, whereas slight restoration was also seen in TLCK- and TPCK-mediated inhibition (Fig. 7). The restoration was abolished when the cells were preincubated adequately with the inhibitors. The restoration from PMSF-mediated inhibition was also abolished when the cells were preincubated with 1 mM PMSF for 40 min at $37^\circ C$ (data not shown). The similar results were also obtained in the monocyte O_2^- production (Fig. 7).

DISCUSSION

Recent reports indicate that human peripheral blood monocytes, like PMN, release O_2^- during phagocytosis, on stimulation by phorbol myristate acetate and on contact with fixed aggregated immunoglobulin (Ig)G (25, 26). As shown in this study, contact with the surface-active agents, Cyt E and Con A, also stimulated not only human PMN (18, 19) but also human monocytes to release O_2^- , although some differences of the pattern and sensitivity of response to the stimuli were seen between PMN and monocytes. These differences indicate that the O_2^- -producing system, in-

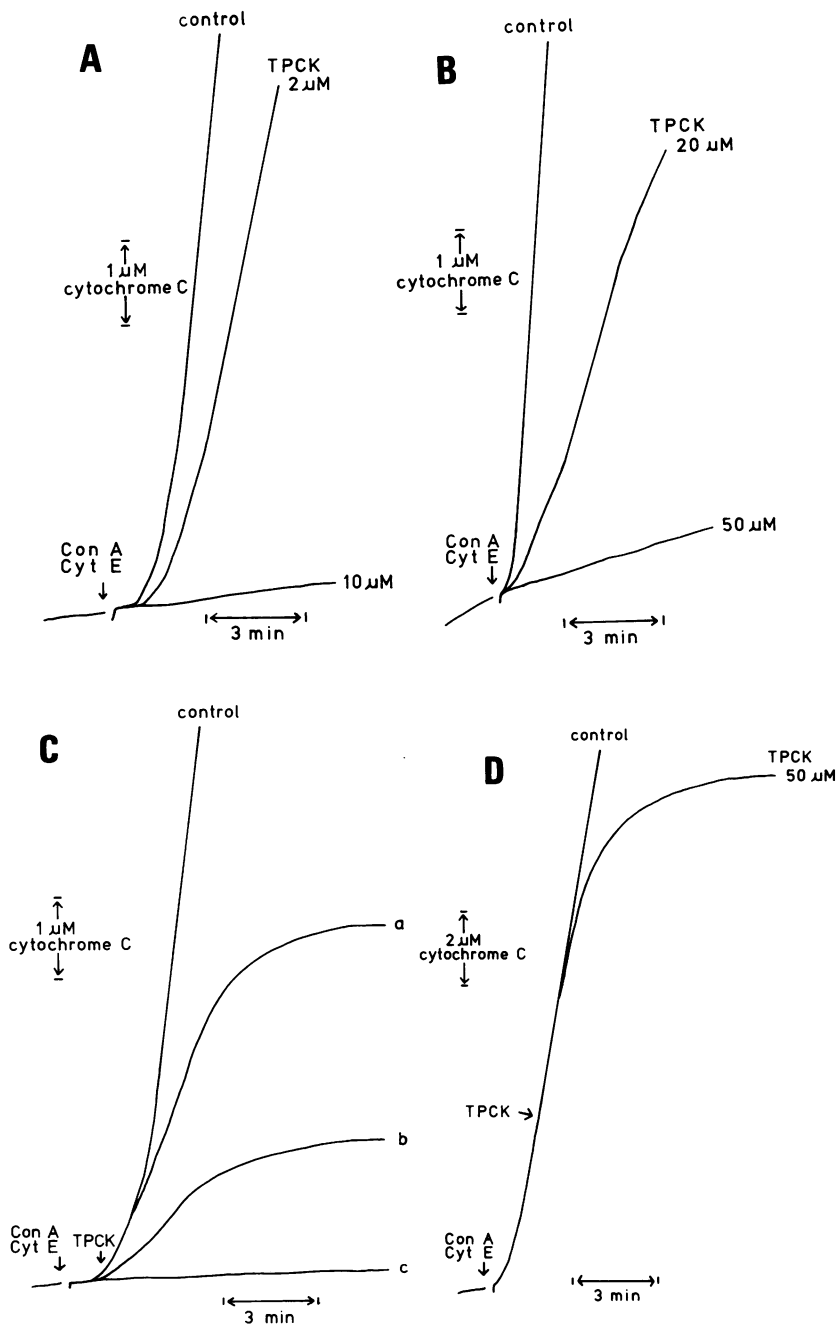


FIGURE 4 The inhibitory effect of TPCK on the O_2^- production by human PMN and monocytes. Cell suspensions, (A) PMN, 2×10^5 /ml, and (B) mononuclear cells, 2×10^6 /ml, were preincubated with TPCK for 3 min at 37°C before Cyt E ($5 \mu\text{g/ml}$) and Con A ($50 \mu\text{g/ml}$) were added. (C) TPCK ($10 \mu\text{M}$) was added to the reaction mixture containing PMN (2×10^5 /ml) (a) 1 min after (during the induction time), (b) at the same time: or (c) 5 min before the addition of Cyt E ($5 \mu\text{g/ml}$) and Con A ($50 \mu\text{g/ml}$). (D) TPCK was added at the time of maximum O_2^- production by mononuclear cells (1.75×10^6 /ml) stimulated by Cyt E ($5 \mu\text{g/ml}$) and Con A ($50 \mu\text{g/ml}$).

cluding the surface membrane, of human PMN may be different from that of human monocytes. Con A binds to the specific sugars on the cell surface membrane

and recent reports suggest that cytochalasins also bind to the cell surface membrane (27, 28).

It is unknown how Cyt E and Con A are able to in-

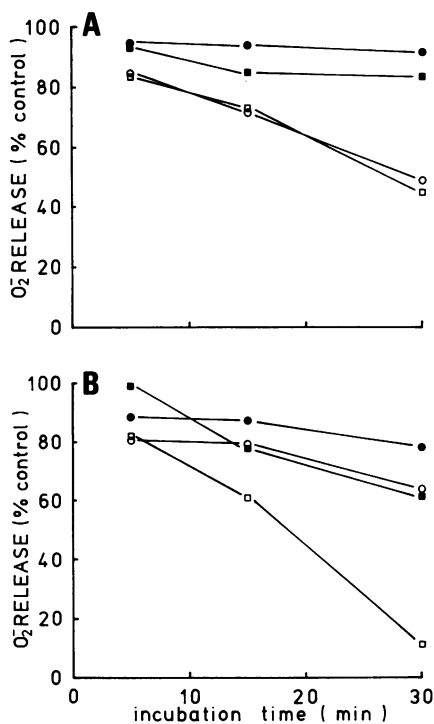


FIGURE 5 The effect of preincubation time with TPCK or PMSF on O₂⁻ production by human PMN (A) and monocytes (B). Cell suspensions were preincubated with TPCK or PMSF for the indicated periods at 37°C before Cyt E (5 μg/ml) and Con A (50 μg/ml) were added. (A) ●, 100 μM PMSF; ○, 250 μM PMSF; ■, 0.2 μM TPCK; □, 0.5 μM TPCK. (B) ●, 250 μM PMSF; ○, 500 μM PMSF; ■, 2.5 μM TPCK; □, 5 μM TPCK.

duce the marked enhancement of the O₂⁻ production by human PMN and monocytes when added simultaneously. We have recently found that *N*-formylmethionyl peptides, which bind to the specific receptor sites on the surface membrane (29), can induce the O₂⁻ production by human PMN and monocytes, and that *N*-formylmethionyl peptide induced O₂⁻ production is markedly enhanced by Con A as well as Cyt E.³ The stimulation of the oxidative metabolism of the phagocytic cells are suggested to be provoked by the membrane perturbation that may result from the surface redistribution of the ligand-receptor complexes (30). From our associated experiments,³ we have suggested that Con A-receptor complexes and Cyt E-receptor complexes may interact on the surface membrane and perturb the surface membrane effectively to activate the NAD(P)H oxidase, resulting in the marked enhancement of the O₂⁻ production. These interactions on the cell surface membrane may con-

³ Kitagawa, S., F. Takaku, and S. Sakamoto. A Comparison of the Superoxide Releasing Response in Human Polymorphonuclear Leukocytes and Monocytes. Submitted for publication.

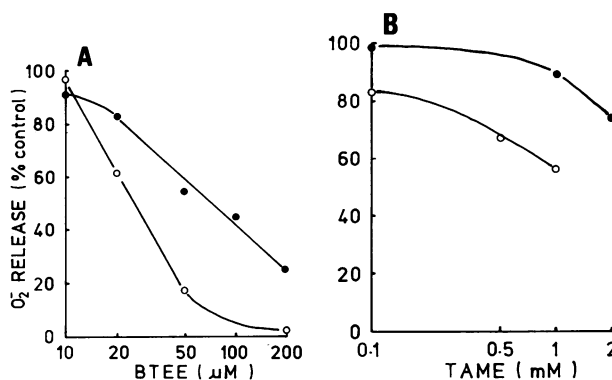


FIGURE 6 The inhibitory effect of synthetic substrates for serine proteases BTEE (A) and TAME (B) on O₂⁻ production by human PMN and monocytes. Cell suspensions were preincubated with various concentrations of BTEE or TAME for 10 min at 37°C before Cyt E (5 μg/ml) and Con A (50 μg/ml) were added. ○, PMN; ●, monocytes.

tribute to the marked enhancement of the O₂⁻ production induced by Cyt E and Con A, used in these experiments.

The O₂⁻ production by human PMN and monocytes was inhibited by the inhibitor and substrate for chymotrypsin-type protease (TPCK and BTEE) as well as those for trypsin-type protease (TLCK and TAME), although the inhibitory effect of the former was much greater than that of the latter (9, 16, 23, 24). It has been reported that TPCK is a specific inhibitor of chymotrypsin, and that TLCK is a specific inhibitor of trypsin (11–13). However, the selectivity of these inhibitors

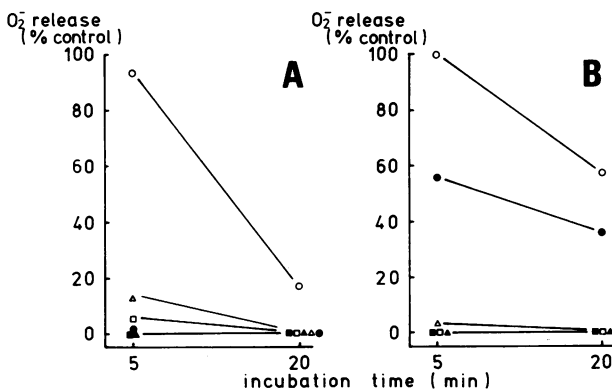
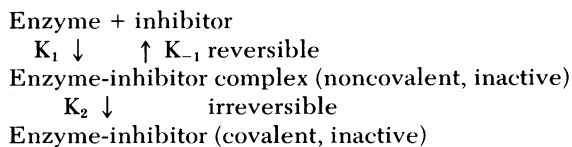


FIGURE 7 Restoration of PMN (A) and monocyte (B) O₂⁻ production by removal of protease inhibitors. Solid symbols indicate O₂⁻ production by human PMN and monocytes that were preincubated with the inhibitors for 5 and 20 min at 37°C and were not washed. Open symbols indicate O₂⁻ production by human PMN and monocytes that were preincubated with the inhibitors for 5 and 20 min at 37°C and were washed twice to remove the inhibitors from the milieu. Control cells were preincubated with the same concentration of dimethyl sulfoxide and simultaneously run. O₂⁻ production was induced by Cyt E (5 μg/ml) and Con A (50 μg/ml). (A) ○, ●, 1 mM PMSF; □, ■, 10 μM TPCK; △, ▲, 500 μM TLCK. (B) ○, ●, 1 mM PMSF; □, ■, 50 μM TPCK; △, ▲, 500 μM TLCK.

may not be absolute but may be only a quantitative difference (11, 31). This may partly explain that the O_2^- production was inhibited not only by TPCK but also by TLCK. The O_2^- production was sufficiently inhibited by ester substrate for chymotrypsin-type protease (BTEE), indicating that the esterase activity may contribute to the O_2^- production (23, 24). The O_2^- production was also inhibited by macromolecular inhibitors (aprotinin and SBTI). These above findings indicate that the proteases involved in the O_2^- production by human PMN and monocytes are similar to chymotrypsin rather than trypsin, and located at the cell surface membrane (9). The serine protease inhibitors were able to inhibit the O_2^- production when added at any time, indicating that enzymatically active serine proteases are essential for human PMN and monocytes to initiate and maintain the O_2^- production in response to the stimuli.

It is unlikely that the relative resistance of monocytes to protease inhibitors results from the contaminating lymphocytes and the large cell number of the mononuclear cell fraction. There may be two possibilities; (a) proteases involved in the O_2^- production by monocytes may not be identical to those of PMN; (b) differences of the O_2^- -producing system, including the surface plasma membrane, between PMN and monocytes, which also may explain the differences of the responsive pattern to the same stimuli. From our present experiments, it is difficult to explain with certainty why the inhibition profile of the O_2^- production in PMN is different from that in monocytes.

If the serine proteases involved in the O_2^- production exist in an enzymatically active form, it would be expected that the restoration of the O_2^- production could not appear even if PMSF, TPCK, or TLCK were removed from the milieu by a simple washing procedure after the preincubation with the cells. The effects of these serine protease inhibitors are irreversible because they ultimately form covalent bonds at the active sites of the enzymes (10, 11). On the other hand, if the serine proteases exist in an enzymatically inert form (proenzyme) and become activated on contact of cells with stimulants, the restoration of the O_2^- production would be possible when the inhibitors are removed from the milieu. However, it has been demonstrated that PMSF, TPCK, or TLCK form a reversible complex as an intermediate with chymotrypsin or trypsin, as shown in the following formula (10, 11).



If these reactions should also occur between the inhibitors and the enzymatically active serine proteases

involved in the O_2^- production, the restoration might be explained by the existence of a reversible complex, which is, however, enzymatically inactive. In addition, the differences of the restoration rate from PMSF-, TPCK-, and TLCK-mediated inhibition would be suggested to reflect the differences of the velocity constants (K_1 , K_{-1} , and K_2) of the reaction between each inhibitor and the serine protease. Furthermore, the restoration was abolished when the cells were preincubated adequately with the inhibitors, indicating that the serine proteases involved in the O_2^- production possibly exist in an enzymatically active form but not in an inert form. It appears that our present results support the reactions shown in this formula.

Recent reports indicate that the O_2^- -producing system, including NAD(P)H oxidase, is possibly located at the outer surface membrane (22, 32–34). Our present results also provide additional evidence that the O_2^- -producing system may be located at the cell surface membrane. NAD(P)H oxidase, the primary enzyme for O_2^- production, may be activated by the interaction of the appropriate stimuli and the cell surface membrane. It is unknown how the serine proteases might be involved in the activation of NAD(P)H oxidase. There may be two possibilities. (a) If the precursor of NAD(P)H oxidase would be a natural substrate for the serine proteases, the precursor might gain access to the proteases by conformational changes of the surface membrane induced by stimuli and might be activated. (b) The serine proteases might be associated with the movement of the macromolecules in the surface membrane; as it has been suggested that the triggering of the metabolic activation of phagocytic cells is provided by a surface redistribution of the ligand-receptor complexes, which may perturb the surface membrane to activate the NAD(P)H oxidase (30). If this is the case, it may be possible that impaired movement of the macromolecules in the surface membrane may also contribute to the inhibition of chemotaxis and phagocytosis in addition to O_2^- production by serine protease inhibitors, inasmuch as chemotaxis and phagocytosis also accompany the movement of the macromolecules in the surface membrane (35–37).

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