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THE EFFECT OF SYNTHETIC PROTEASE INHIBITORS ON LYSOSOMAL ENZYME RELEASE FROM HUMAN NEUTROPHILS

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THE EFFECT OF SYNTHETIC PROTEASE INHIBITORS ON LYSOSOMAL ENZYME RELEASE FROM HUMAN NEUTROPHILS

By

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ABSTRACT

THE EFFECT OF SYNTHETIC PROTEASE INHIBITORS ON LYSOSOMAL ENZYME RELEASE FROM HUMAN NEUTROPHILS

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Serine esterase activity has been implicated in the neutrophil function of lysosomal enzyme release. To better characterize this esterase role and its possible plurality, the effect of synthetic protease inhibitors on complement- and noncomplement-mediated enzyme secretion was investigated.

Noncytotoxic, selective degranulation was achieved by exposing human neutrophils to either 10% Zymosan-Activated Plasma (ZAP) or 10^{-4}M N-formyl-Methionyl-Phenylalanine (fMP) in the presence of 5 µg/ml of Cytochalasin B. The majority of the releasing capability generated with ZAP was shown to be C5-related using C5-deficient plasma. Lysosomal markers assayed were Beta-Glucuronidase for primary granule response and Lysozyme for secondary granule involvement. The inhibitors were used to pretreat the cells or were added concomitantly with the releasing stimulus. The tryptic inactivator TLCK inhibited both primary and secondary granule release regardless of the stimulus and inactivation protocol. The chymotryptic inhibitor, TPCK, only blocked fMP-induced

release in the absence of inducer; however, when present with either stimulus, secondary granule secretion alone was inhibited. The sulfonyl halide, PMSF, did not impede release under any of the experimental conditions. The chymotrypsin model substrate, BTEE, blocked secondary release by both ZAP and fMP only when present with the secretory stimuli. Its counterpart for tryptic activity, TAME, had no effect on release.

These studies suggest the involvement of tryptic and chymotryptic activated esterases in lysosomal enzyme release from human neutrophils. Their role may be discriminatory with regard to both the releasing stimulus and granule response.

To Christine and Jennifer whom I dearly love

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LIST OF ABBREVIATIONS

BCF	Bacterial Chemotactic Factor
BG	Beta-glucuronidase
BTEE	Benzoyl Tyrosine Ethyl Ester
ClINA	Cl inactivator
CB	Cytochalasin B
DFP	Diisopropylfluorophosphate
EACA	Epsilon Amino Caproic Acid
fMP	N-formyl-Methionyl-Phenylalanine
HBSS	Hank's Balanced Salt Solution
LDH	Lactic Dehydrogenase
LYS	Lysozyme
PMN	Polymorphonuclear leukocyte
PMSF	Phenylmethylsulfonylfluoride
TAME	Tosyl Arginine Methyl Ester
TLCK	N-tosyl-L-lysyl-chloromethyl-ketone
TPCK	N-tosylamide-phenylethyl-chloromethyl-ketone
ZAP	Zymosan-activated plasma

INTRODUCTION

An inflammatory reaction culminates the body's cellular response to an infectious challenge; however, during the process the protective function of the neutrophil is overshadowed by the deleterious effect of its lysosomal constituents on the surrounding tissues (18,22,34). Enzymes are released either during phagocytosis by regurgitation during feeding or when the cells encounter a non-phagocytosable surface by reverse endocytosis (69). Factors which are chemotactic for neutrophils will also promote lysosomal degranulation. These include complement-derived factors such as the active fragments of the third and fifth components of complement, C3a and C5a (10,11) as well as serum or plasma activated through the alternate complement pathway by zymosan (27): a bacterial chemotactic factor (BCF) and synthetic di- and tripeptides formylated at their N-termini (10,55). The release induced by these chemotactic substances is enhanced significantly when the neutrophils are exposed to the fungal metabolite Cytochalasin B (27,55). This drug disrupts contractile microfilament function and inhibits chemotaxis as well as phagocytosis at concentrations that cause increased lysosomal secretion (9,75,76). Thus, the use of CB-treated neutrophils provides

a model system for the study of lysosomal degranulation in the absence of phagocytosis. By their inhibition with DFP, the neutrophil functions of chemotaxis, phagocytosis and lysosomal enzyme release demonstrate the role of one or more serine esterases in the activation of these inter-related processes (11,51,67). The synthetic inhibitors TPCK and TLCK, specific for chymotrypsin-like and trypsin-like esterases, respectively, irreversibly inactivate the chemotactic response to C3a and C5a (2,25). These inhibitors also block the ability of neutrophils to hydrolyze chemotactic peptides, while inhibiting neutrophil directed migration to C5a, BCF, and fMP. The chemotactic response to these factors is competitively inhibited by the model substrate for chymotrypsin activity, BTEE (3). Since correlation coefficients are high between chemotaxis and lysosomal secretion when cells are stimulated by N-formylated synthetic peptides (13), perhaps these cell functions demonstrate different or similar inhibitory responses to more specific proteinase inhibitors.

The purpose of this study is to discern the effect of certain proteinase inhibitors on the lysosomal enzyme release of CB-treated human neutrophils stimulated with ZAP and fMP. This may further delineate the characteristics of the serine esterase involvement with this neutrophil process.

LITERATURE REVIEW

Neutrophil Lysosomes

The normal polymorphonuclear leukocyte (PMN) contains two granule types based upon staining with Wright-Giemsa stain which is used routinely for making peripheral blood smears. During granulopoietic maturation, azurophilic (primary) granules are formed first at the promyelocyte stage while the specific (secondary) granules are not found until the more mature myelocyte stage. Two-thirds of the granules of the mature PMN are specific with the remainder being azurophilic. Cytochemical studies using electron microscopy show that the primary lysosomes contain peroxidase and acid hydrolases such as acid phosphatase and beta-glucuronidase. The secondary lysosomes lack acid hydrolases and peroxidase; however, they do contain alkaline phosphatase, some neutral proteases, and bactericidal substances such as lysozyme (muramidase). One-third of this enzyme's total lysosomal content is also found in the primary granule (5,6). The degranulation of lysosomal enzymes is a secretory process that occurs in a sequential manner with secondary granule secretion preceding primary (7,32). This may be related to the fact that secondary granules are particularly accessible for extracellular release (43). Morphological

studies using a discontinuous sucrose gradient demonstrate a heterogeneity of lysosome populations in rabbit and human PMNs beyond the specific and azurophilic staining of granules. Small, low-density granules which are specific contain alkaline phosphatase and most of the lysozyme activity. The large high-density granules which are azurophilic contain peroxidase, the remainder of the lysozyme activity, and most of the acid hydrolases. A morphologically heterogeneous fraction contains the remainder of the acid hydrolases, but no myeloperoxidase (5,71). A discontinuous sucrose gradient of human neutrophil lysates separates the granules into two high-density azurophilic bands while the specific lysosomes collate into a band of lesser density (74). All of these studies indicate there are two populations of azurophilic lysosomes and one population of specific granules. Besides the digestive and bactericidal properties of lysosomal enzymes, they are also capable of interacting with components of the complement system. Incubation of lysosomal enzymes from human PMNs with C1, C3, or C5 at a neutral pH has shown alterations usually associated with the sequential immunologic activation of the complement system (61). C1 esterase can be activated (61) and then subsequently inactivated (62) with incubation. C3 is cleaved into large and small fragments and more recently it has been shown that human leukocyte elastase cleaves C3 into fragments which can be partially characterized (63). Within the neutrophil lysosomes are

neutral proteases which both activate C5 (68) and inactivate C5a, the chemotactic fragment of C5 (73). Both of these enzymes are found in separate granules and subject to sequential release. The C5a generation activity is found in the specific granules while C5a inactivation capability is contained in the azurophilic granules (74). These studies suggest an interplay between lysosomal enzymes and complement components in the generation and exacerbation of the inflammatory response.

Selective Degranulation

The secretion of lysosomal enzymes from neutrophilic granulocytes involves migration of the granules to the localized area of contact with the foreign substance or surface. Fusion of the lysosomal membrane with the plasma membrane occurs and the enzymes are released into a preformed vacuole in actual phagocytosis or to the cell exterior in "frustrated" phagocytosis, i.e., no ingestion (69). This degranulation with leakage or release of enzymes to the cell exterior causes tissue damage (19,22,34). The most injurious constituents are proteases, collagenase, elastase, and permeability factors--all capable of digesting and injuring tissues. Peripheral blood leukocytes contain IgG receptors as well as trypsin-sensitive complement receptors for C3 and C5. Neutrophils when exposed to phagocytosable and nonphagocytosable immune complexes selectively release their enzymes in a noncytotoxic, noncytolytic manner (30,32,69). In human PMNs, the selective

release caused by complex uptake resembles that induced by zymosan particle ingestion; both particle and complex uptake are accompanied by an increase in hexose monophosphate shunt activity (69). Aggregated human myeloma proteins of all IgG and IgA subclasses and normal IgG react with human neutrophils in a serum-free medium causing enzyme release. The other immunoglobulin classes (IgD, IgE, IgM) do not stimulate degranulation. The insoluble aggregates adhere to the neutrophils, are ingested, and concomitantly enzymes leak to the cell exterior prior to vacuole closure. Soluble aggregates do not induce secretion when in suspension with neutrophils but do effect secretion when bound to a nonphagocytosable surface (35,36). Under the appropriate conditions substances which are chemotactic for neutrophils may also induce enzyme release. The complement-derived factors include the active fragments of the third and fifth components of complement, C3a and C5a (10,11), and serum or plasma activated through the complement alternate pathway by zymosan (27). C3a, C5a, and a bacterial chemotactic factor (BCF) are capable of activating Proesterase I in rabbit neutrophils. Activation of this proenzyme is believed to be an obligatory step in the chemotactic activity induced by these substances (8). The surface charge of human granulocytes is diminished when incubated with C5a. This suggests that a decrease in cell surface charge is a prerequisite for normal cell movement (24). Synthetic peptides formylated at the N-termini are

strong attractants for neutrophils and macrophages (54). These di- and tripeptides can also induce enzyme release and their potency is greatest when the hydrophobic amino acid phenylalanine is positioned at the C-terminus (55). A statistical and linear correlation exists between enzyme release and the chemotactic response evoked by these peptides. A higher peptide concentration is needed to induce enzyme secretion than the corresponding chemotactic response (13,55). Specific receptor sites for chemotactic peptides have been demonstrated on rabbit as well as human neutrophils and the binding is rapid yet reversible (^{3},72). Other effects can be observed with neutrophils upon exposure to chemotactic factors. The rate of ingestion of sheep erythrocytes sensitized with IgG and complement is reduced significantly with C5a, BCF, and the peptide formyl-Methionyl-Leucine (47). An excellent correlation exists between calcium influx and lysosomal enzyme release. In addition, a large and rapid potassium efflux is observed under conditions which give rise to enzyme secretion using the peptide formyl-Methionyl-Leucyl-Phenylalanine (fMLP). This suggests a possible ionic basis for degranulation (48). In rabbit PMNs, fMLP stimulates protein carboxymethylation but not protein synthesis, revealing a high correlation between chemotactic responsiveness and specific carboxymethylation (50).

Cytochalasin B (CB)

This biologically active mold metabolite is obtained from the fungus *Helminthosporium dematoideum*. The literal translation of cytochalasin is "cell relaxation" and this was the original description of its characteristic effect and not its mode of action (1,17). Cytochalasin B has a unique and novel macrolide structure in which a lactone ring is joined to a bicyclic lactam system. Cultured mammalian cells that have been exposed to CB show an increased rate of attachment to glass, a reversible inhibition of cell locomotion, and total inhibition of cytoplasmic cleavage, without interfering with nuclear division during mitosis. Although multinucleated, these cells remain viable for days in the presence of cytochalasin B (17). Human neutrophils and rabbit macrophages show a disruption and depletion of microfilaments when treated with cytochalasin B (46). The influence of CB on neutrophil functions is reversible and diverse. Phagocytosis (16,21,46,75) and chemotaxis (9,15,75) are either stimulated or inhibited by cytochalasin B depending on the drug's concentration. Rabbit and human PMNs show suppression and eventual inhibition of both particle and bacteria uptake at CB concentrations above 5 µg/ml (16,75). When dropped below 1 µg/ml, CB stimulates the uptake of both live staphylococci and starch particles in human neutrophils (16). A similar pattern exists with the PMN chemotactic response. At a concentration range of 2 to 4 µg/ml, CB reversibly inhibits the directed migration

and locomotion of rabbit and human PMNs (9,75); however, below 1 μg/ml chemotactic stimulation is generated (9). Lysosomal enzyme release is enhanced with CB regardless of the inducing stimulus (11,20,37,76), including phagocytosable and nonphagocytosable substances. The enhanced release occurs above a 5 μg/ml concentration of CB with a maximal effect at 10 μg/ml (37,76). Another study has shown that this drug delays and decreases primary granule release stimulated by zymosan particles at 10 μg/ml (57). In any case, CB at its highest concentration does not affect the total activities of assayed lysosomal enzymes even for incubation periods of 72 hours (20). Electron micrograph examination of CB-treated human neutrophils shows nuclear and cytoplasmic spreading with a linear arrangement of the granules. One hypothesis for the enhancing effect that CB has on enzyme release is the removal of normal constraints for the merger of granules with each other or the plasma membrane (76). Besides its effects on the neutrophil functions of phagocytosis, chemotaxis, and lysosomal enzyme release, CB also strongly inhibits sugar uptake (75), oxygen consumption and hexose monophosphate shunt activity (37,52). The fungal metabolite enhances phospholipid metabolism in guinea pig peritoneal leukocytes. In fact, kinetic studies show that the drug's influence on the increased incorporation of inorganic phosphorus into phosphatidic acid and phosphoinositides and the enhanced release of beta-glucuronidase are comparable.

The increased synthesis of phosphoinositides in CB-treated cells may interact with cationic protein and facilitate the fusion of lysosomal and plasma membranes (64). Studies have shown that chemotactic factors increase PMN volume; however, the cell volume decreases when the cells are exposed to CB alone or CB with a chemotactic factor. There is no quantitative correlation between this phenomenon and the secretion of lysosomal enzymes (41,42). Microtubules have been implicated in granule movement and lysosomal enzyme release (28). Colchicine disrupts or eliminates microtubules which are demonstrable in mature PMNs (45); it also inhibits enzyme secretion and microtubule assembly in a dose-related fashion (40). Concanavalin-A, a plant lectin and lymphocyte mitogen, induces microtubule assembly and specific granule discharge in human PMNs (39). Thus, the importance of microfilaments and/or microtubules is apparent with regard to lysosomal enzyme release. The use of CB-treated neutrophils provides a model system for the study of selective degranulation in the absence of phagocytosis.

Inhibition of Neutrophil Functions

As noted earlier, the inflammatory functions of the neutrophil involve an energy source, calcium and/or magnesium in the external medium, and possible involvement of the contractile elements of the cell. Any substance which may inhibit or block any of these parameters may obstruct any or all of the neutrophil functions concerned with inflammation.

Nonphagocytic lysosomal enzyme release is inhibited by cAMP and theophylline but the effect in combination is no greater than additive. The anti-inflammatory agents hydrocortisone and colchicine are also inhibitory (31,77). Of particular interest is the effect that phosphonate esters have on PMN functions, especially the inhibitor diisopropylflurophosphate (DFP). This irreversible inactivator functions through phosphorylation of the serine hydroxyl group within the active site of the affected enzyme. The charge relay between the serine, aspartic acid and histidine residues within the active site is blocked, thereby rendering the serine esterase nonfunctional. In studies done with rabbit neutrophils, DFP was shown to inhibit chemotaxis whether the cells were pretreated or the inhibitor was present with the chemotactic factor. The same inhibitory effect seen under different conditions suggests that two esterases--one activated and the other activatable--are in or on the cell and are involved in the chemotactic response. This is reinforced by the difference in inhibition profiles exhibited by either cell-dependent or chemotactic factor-dependent inactivation (12,67). One approach toward observing phagocytosis is treatment of guinea pig peritoneal cells with EAC1423 using sheep erythrocytes and guinea pig serum as a complement source. This complement-dependent erythrophagocytosis is inhibited by DFP. The effect is irreversible, temperature-dependent, and proportional to the inhibitor

concentration and the duration of exposure to the inhibitor. Cell pretreatment with DFP gives the greatest effect, suggesting that a critical proteinase or esterase is in or on the cell in the "activated" state and a second activatable enzyme is also required for the phagocytic process (51). Lysosomal enzyme release from rabbit neutrophils by BCF is inhibited with DFP, but only when the inhibitor and chemotactic factor are together and not when the cells are treated before release (11). Human neutrophil degranulation using zymosan particles (phagocytic stimulus) or aggregated IgG bound to a surface (nonphagocytic stimulus) is inhibited by DFP. Some effect is seen when the cells are preincubated with the inhibitor prior to release; however, the strongest result occurs with the stimulus and inhibitor together (38). The observations with DFP show that serine esterase activity is a critical part of chemotaxis, phagocytosis, and enzyme secretion. This has led to studies with more specific, irreversible serine esterase inhibitors such as N-tosyl-L-lysyl-chloromethyl-ketone (TLCK), N-tosylamide-phenylethyl-chloromethyl-ketone (TPCK), phenyl-methyl-sulfonyl-fluoride (PMSF), C1 inhibitor (C1INH), and alpha-1-antitrypsin. C1INH is a biological broad-spectrum inactivator exhibiting control over the coagulation, fibrinolysis, complement, and kallikrein blood systems and it weakly inhibits the pancreatic enzymes trypsin and chymotrypsin. Alpha-1-antitrypsin is also a biological proteinase inhibitor that blocks the fibrinolytic and

kallikrein systems, but it strongly inactivates both trypsin and chymotrypsin (33). The synthetic serine esterase inhibitors TPCK and TLCK function by alkylation of the histidine residue within the active site of the enzyme. Functioning as pseudosubstrates, TLCK inactivates chymotrypsin-like proteinases while TPCK affects trypsin-like proteinases. The synthetic inhibitor PMSF functions like DFP; however, it sulfonylates the serine hydroxyl in the enzyme's active site. In contrast to the organophosphates, sulfonyl halides show great differences in reactivity depending on the enzyme and the structure of the sulfonyl halide (26). Fibroblasts that have been treated with Simian Virus 40 (SV-40) lose their ability to exhibit contact inhibition and consequently grow proliferatively in tissue cultures. Treatment of these transformed cells with TPCK inhibits their growth; an identical "growth plateau" effect is seen also with cycloheximide, an inhibitor of *de novo* protein synthesis in eukaryotes. This suggests that the growth inhibitory effect of TPCK on transformed cell growth involves the inhibition of protein synthesis instead of proteolytic inactivation (18). TLCK, TPCK, and PMSF inhibit the attachment of Baby Hamster Kidney (BHK) cells to a substratum only in the presence of serum; that is, significant inhibition occurs when the substratum is coated with serum. The effect is achieved by preincubating the cells with the inhibitor at room temperature; after washing the cells are resuspended in fresh attachment medium. Under these conditions, the

findings demonstrate that the cellular function of attachment also requires cell-related esterase activity (29). Human C1INH reversibly enhances human neutrophil chemotactic response to activated plasma or serum without affecting spontaneous motility (59). This same proteinase inactivator also shows chemotactic inhibition to trypsin-activated C5 while significantly enhancing the response to N-formyl-methionyl-phenylalanine (44). These effects with C1INH occur only when the inhibitor is present with the cells in the upper portion of the Boyden chamber. Preincubation of human neutrophils with TLCK, TPCK, and alpha-1-antitrypsin inhibits their chemotactic responsiveness to C3a and C5a. TPCK inhibits random migration while TLCK and alpha-1-antitrypsin enhance it (25). The potencies of fMet peptides as chemotactic agents is related to the rate at which they are hydrolyzed. TPCK and TLCK inhibit chemotaxis as do the hydrolysis products of fMet peptides. Neutrophils pretreated with TPCK and TLCK inhibit peptide hydrolysis and the chemotactic response to C5a, BCF, and fMP. In addition, benzoyl tyrosine ethyl ester (BTEE), a model substrate specific for chymotrypsin activity, inhibits the chemotactic response to all three attractants. Tosyl arginine methyl ester (TAME), which is used to identify trypsin-like activity, has little or no effect on chemotaxis (2). Rabbit neutrophils have chymotrypsin-like esterase activity in their cytosol and lysosomes. The esterase is not inhibited by cAMP, nor is it stimulated by

BCF or C5a. High chemotactic activity is found in the partially purified fraction of the enzyme. Both chemotactic and enzymatic activities are inhibited by a phosphonate ester (65). Unlike rabbit PMNs, essentially all the chymotrypsin-like esterase activity of human neutrophils is in the lysosomal fraction, but more specifically the majority is in the primary granule. Human esterase activity has a sharper optimum pH range and the specific activity may be as high as 40-fold that observed in rabbit (66). Since the neutrophil functions of chemotaxis, phagocytosis, and lysosomal enzyme release require one or more surface esterases, a suggestive activation scheme can be hypothesized. Interaction of a chemotactic factor with a specific receptor could activate the surface esterase(s) leading to interdependent events causing chemotaxis or enzyme release (12,13). Included in these interrelated events could be a transient increase in membrane permeability to sodium and calcium, membrane depolarization and the involvement of microfilaments and/or microtubules (48).

MATERIALS AND METHODS

Reagents and Solutions

Sodium Hypaque (50%) was obtained from Winthrop Laboratories, New York, NY 10016. Triton X-100 was purchased from Research Products International Corporation, Elk Grove Village, IL 60007; Goat anti-human C5, Meloy Laboratories, Alexandria, VA; Protein A-Sepharose, Pharmacia Fine Chemicals, Piscataway, NJ. The remaining materials were procured from Sigma Chemical Company, St. Louis, MO 63178. These included bovine serum albumin (BSA), human serum albumin (HSA), benzoyl-tyrosine ethyl ester (BTEE), Cytochalasin B (CB), cycloheximide, Dextran, dimethylsulfoxide (DMSO), Epsilon Amino Caproic Acid (EACA), Ficoll (MW 400,000), Lysozyme Egg White Standard, *Micrococcus lysodeikticus*, N-formylmethionylphenylalanine (fMP), β-nicotinamide adenine dinucleotide (β-NADH), phenolphthalein beta-glucuronic acid, phenylmethylsulfonylfluoride (PMSF), tosyl-arginine methyl ester (TAME), L-tosylamide-phenylethylchloromethyl ketone (TPCK), N-tosyl-L-lysine chloromethyl ketone (TLCK), and zymosan A.

A stock solution of Cytochalasin B (5 mg/ml in DMSO) was kept at 4°C. The stock solution was diluted with Hank's Balanced Salt Solution, pH 7.4 (HBSS) to a concentration of

50 µg/ml. Aliquots of 10^{-3}M fMP in HBSS and 10 mg/ml BSA in HBSS were stored at -20°C until used. TLCK and cycloheximide readily dissolved in HBSS to the desired concentrations. TPCK and PMSF did not go into aqueous solution below 10^{-3}M. They were therefore dissolved in anhydrous methanol at 0.1M and then diluted with HBSS to concentrations of 10^{-3}M or less. Ficoll-Hypaque Solution was made by combining 24 parts of 9% Ficoll with 10 parts of 33.9% Hypaque. Human C5 was isolated according to Nilsson (49) without modification.

Neutrophil Isolation

Heparinized whole blood was obtained from healthy human volunteers. Nine parts of blood was mixed with 1 part of 6% Dextran (in HBSS) and allowed to settle for 1 hour. Four milliliters of the cell-rich plasma was layered over 4 ml of Ficoll-Hypaque Solution in siliconized 16 x 100 mm glass tubes. After centrifugation at 800 x g for 20 minutes, the polymorphonuclear cells pelleted to the bottom along with some erythrocytes. The supernatant which contained mononuclear cells and platelets was discarded. The cell pellet was suspended in HBSS, transferred to a clean siliconized tube, and washed once. Cell counts were accomplished on a hematocytometer with the white cell concentration being adjusted to 20 x 10^6 cells/ml. These preparations contained approximately 95% neutrophils with an estimated red to white cell ratio of 5:1 (58). Aliquots (0.1 ml) of the cell suspension were placed in 10 x 75 mm

glass tubes. The final reaction volume and cell concentration throughout experimentation was 2 x 10^6 cells/ml/tube.

Enzyme Assays

Lactic Dehydrogenase (LDH), the cytoplasmic marker for cell viability, was determined by the method of Bergemeyer et al. (14). This bisubstrate reaction measured the change in absorbance at 366 nm due to the oxidation of NADH. The absorbance decrease of the 3-ml reaction mixture was observed over a 2-minute interval. Enzyme activity was tabulated and recorded as units/ml.

Lysozyme, the secondary granule marker, was measured according to the Shugar method as described in the Sigma Bulletin for Lysozyme Egg White Standard (56). This assay also involved a change in absorbance over a fixed time period. One-tenth milliliter of the unknown was added to a 2.5 ml suspension of *Micrococcus lysodeikticus* and the decrease in absorbance at 450 nm after 2 minutes was recorded. The enzyme's activity was expressed as units/ml.

The primary granule indicator, β-Glucuronidase, was assayed by the Fishman method (23). A sample of the unknown (0.1 ml) was added to 0.1 ml of phenolphthalein glucuronic acid and 0.8 ml of acetate buffer making the final reaction volume and pH 1.0 ml and 4.5, respectively. After 17 hours of incubation at 37°C, the solution was made alkaline with a glycine buffer (pH 11.2), trichloroacetic acid and water. The phenolphthalein released due to substrate hydrolysis caused the development of a pink color

whose intensity was dependent on the enzyme concentration. The solution's absorbance was measured at 540 nm and compared to 25 µg/ml and 50 µg/ml standards made by diluting in HBSS aliquots from a stock phenolphthalein solution of 1 mg/ml in ethanol. BG values were reported as µg phenolphthalein/ml.

Enzyme Secretion Protocol

Stimulated release of primary and secondary neutrophil granules was effected with ZAP and fMP in the presence of 5 µg/ml cytochalasin B (13,27).

A suspension of zymosan A (10 mg/ml) was made in phosphate-buffered saline, pH 7.4, ionic strength 0.15, containing 0.15M magnesium chloride. One-tenth milliliter of the zymosan suspension was added to 0.9 ml autologous plasma containing .25M EACA. In our hands EACA allowed substantially greater enzyme release than plasma alone when utilizing ZAP, as previously shown by Goldstein et al. (27). After incubation at 37°C for 30 minutes the zymosan particles were removed by centrifugation at 1000 x g for 5 minutes. One-tenth milliliter of ZAP and 0.1 ml CB (50 µg/ml) were added to 0.8 ml HBSS containing 2 x 10^6 cells. After mixing, the suspension was incubated at 37°C for 30 minutes. Supernatants were removed for enzyme assays after centrifugation at 1000 x g for 1 minute.

Noncytotoxic enzyme release occurred with ZAP alone and CB alone; however, together their effect was significantly more than additive. A ZAP dilution control (1:10 in

HBSS) in the absence of neutrophils was assessed for enzyme levels in order to negate any effects due to plasma alone.

When utilizing the formylated peptide, 2×10^6 cells in 0.7 ml HBSS were treated with 0.1 ml BSA (10 mg/ml) and held at 4°C until ready for use. One-tenth milliliter CB (50 µg/ml) and 0.1 ml fMP (10^{-3}M) were then added and mixed. After incubating at 37°C for 5 minutes the supernatants were removed as before and enzyme determinations performed.

Relative secreted enzyme levels were calculated as the percent of total enzyme content of the cells. Total enzyme content was ascertained by treatment of cells with 0.2% Triton X-100. Baseline or unstimulated enzyme levels were assessed on cells incubated in HBSS.

Inhibitor Treatment of Cells

Two approaches were taken to evaluate the effect of inhibitors on enzyme release. The first involved incubation of inhibitor with cells in the presence of the releasing stimulus. In this instance the inhibitor was added to cells, followed immediately by addition of the releasing agent. After an appropriate incubation period (30 minutes for ZAP and 5 minutes for fMP), the supernatants were analyzed for enzyme content. The second protocol utilized preincubation of the cells with inhibitor and a subsequent single washing in HBSS before exposure to ZAP or fMP. The inactivators or inhibitors were added to the PMNs in HBSS, mixed, incubated at 37°C for 5 minutes and centrifuged

(30 seconds at 1000 x g). The supernatants were carefully removed and discarded. The cell pellets were washed once in HBSS and subjected to ZAP or fMP as already described. Control experiments indicated none of the inhibitors elicited enzyme release alone, nor did they inactivate the marker enzymes.

Experiments using TPCK or PMSF required that initial solubilization be effected in anhydrous methanol. Stock solutions of each agent at 0.1M were used for subsequent dilutions in HBSS. Appropriate methanol dilutions did not affect the enzyme assays nor alter stimulated or resting enzyme release.

Statistical Procedure for Data Presentation

Enzyme release values are presented as % $\pm$ Standard Error of the Mean (SEM); 100% designates the total enzyme content determined by treatment with 0.2% Triton X-100. Statistical significance was determined by a paired Student's t-test with n signifying the number of degrees of freedom and p the level of significance. Calculated p values of less than .05 were considered to be statistically significant.

Preparation of C5-Deficient Plasma

Goat antiserum to human C5 was prepared as follows. Twelve precipitin bands were excised from replicate agar immunoelectrophoresis gels which were developed with purified C5 and commercial anti-C5 in the presence of 0.01M EDTA. The precipitin bands were allowed to stand for

several days at 4°C in several changes of physiologic saline containing 0.02% sodium azide. The washed opaque immune precipitates were emulsified in Freund's Complete Adjuvant, and injected subcutaneously into multiple sites in an individual goat. Two weeks later the injection procedure was repeated and 6 weeks after the initial injection the antiserum was harvested, heat inactivated, made 0.02% with sodium azide, and frozen at -20°C or -80°C.

In immunoelectrophoresis (53) and Ochterlony analyses the antiserum was found to possess strong anti-C5, anti-HSA, and anti-IgG activities. If immunodiffusion patterns were allowed to develop for more than 2 days with NHS, an additional faint band was seen that possessed slow beta motility and a slow rate of diffusion. Anti-HSA activity was removed with stoichiometric amounts of HSA and anti-IgG activity was removed by absorption with IgG-Sepharose 6B. The antiserum was rendered monospecific for C5 when observing precipitin bands within 24 hours at room temperature. Moreover, this anti-C5 did not demonstrate reactivity with serum genetically deficient in C5, kindly given to us by Dr. John Leddy.

The IgG fraction of the anti-C5 was isolated by caprylic acid precipitation according to the method of Steinbach and Audran (60). After adjusting the protein content to ∿2%, insolubilization of anti-C5 activity was effected by treatment with Protein-A Sepharose. Packed Protein A-anti C5 contained approximately 20 mg protein/ml resin.

Fifteen milliliters of normal human plasma was treated by stirring for 45 minutes at room temperature with 5 ml of packed resin in the presence of 0.01M EDTA. A second treatment for 90 minutes at 4°C and 30 minutes at room temperature rendered the plasma deficient in C5 as ascertained by immunoelectrophoresis with our anti-C5 as well as with commercial anti-C5. Rocket electrophoresis (4) was also done on the commercial anti-C5. The unidentified precipitating activity described above was still present in the absorbed C5-deficient plasma.

The C5-deficient plasma was dialyzed against HBSS and frozen at -80°C until ready for use. An untreated source of plasma was treated in an identical fashion with regard to incubations, dialysis and freezing. This plasma subsequently served as a positive stimulus control when activated with zymosan.

RESULTS

Inhibition of Enzyme Release with the Trypsin-Like Inhibitor TLCK

When PMNs were treated with TLCK prior to stimulated enzyme release with ZAP and fMP, significant prevention was observed. As shown in Figure 1, both lysozyme and BG release were significantly inhibited. When ZAP was used as the secretory stimulus, BG release was significantly depressed at 10^{-3} and 10^{-4}M TLCK and lysozyme release was inhibited at 10^{-5}M as well. Similar results were obtained when the formylated peptide was used to stimulate granule secretion. In this instance significant inhibition was observed for both granule markers from 10^{-3} to 10^{-5}M TLCK.

Table 1 shows the results of experiments designed to detect inhibition when inhibitor was present simultaneously with either ZAP or fMP. Significant inhibition at 10^{-3}M of both lysozyme and BG was observed when using ZAP. Similar results were obtained with fMP-stimulated cells. Significant inhibition of both enzymes was affected at 10^{-3}M TLCK and BG release was inhibited at 10^{-4}M TLCK as well.

The effect of TLCK on enzyme release is apparently non-cytotoxic since LDH levels remained low throughout these experiments.

Figure 1. TLCK pretreatment. PMNs (2×10^6/ml) were preincubated with the inhibitor for 5 minutes at 37°C, washed once, then treated with (A) 10% ZAP + 5 μg/ml CB with incubation at 37°C for 30 minutes or (B) 10^{-4}M fMP + 5 μg/ml CB + 1 mg/ml BSA with incubation at 37°C for 5 minutes. Results presented as % ± SEM of total enzyme content determined by treatment of $\bar{2} \times 10^6$ cells/ml with 0.2% Triton X-100 (β-Glucuronidase = 128 ± 10 μg phenolphthalein/ml, Lysozyme = 219 ± 13 units/ml, LDH = 388 ± 13 units/ml). Graphic presentation of enzyme levels shown as solid bars (BG), hashed bars (LYS), and open bars (LDH) with tolerances less than 0.5 not displayed. Negative controls for all 3 enzymes with both stimuli were not greater than 5.4 ± .40%. Student's t-test p values for BG and LYS compared to tubes receiving no pretreatment, but subjected to stimuli. LDH p values (n=4), although not significant, were compared to the negative control. In part A, significant inhibition was seen at 10^{-3}M and 10^{-4}M TLCK for both BG and LYS. At both concentrations, $p<.05$ for BG (n=4); for LYS (n=6) $p<.005$ at 10^{-3}M and $p<.02$ at 10^{-4}M TLCK. In part B, significant inhibition was exhibited from 10^{-3}M to 10^{-5}M TLCK. At the higher 2 concentrations, $p<.005$ for both enzymes. At 10^{-5}M TLCK, $p<.05$ for BG (n=4) and $p<.02$ for LYS (n=5).

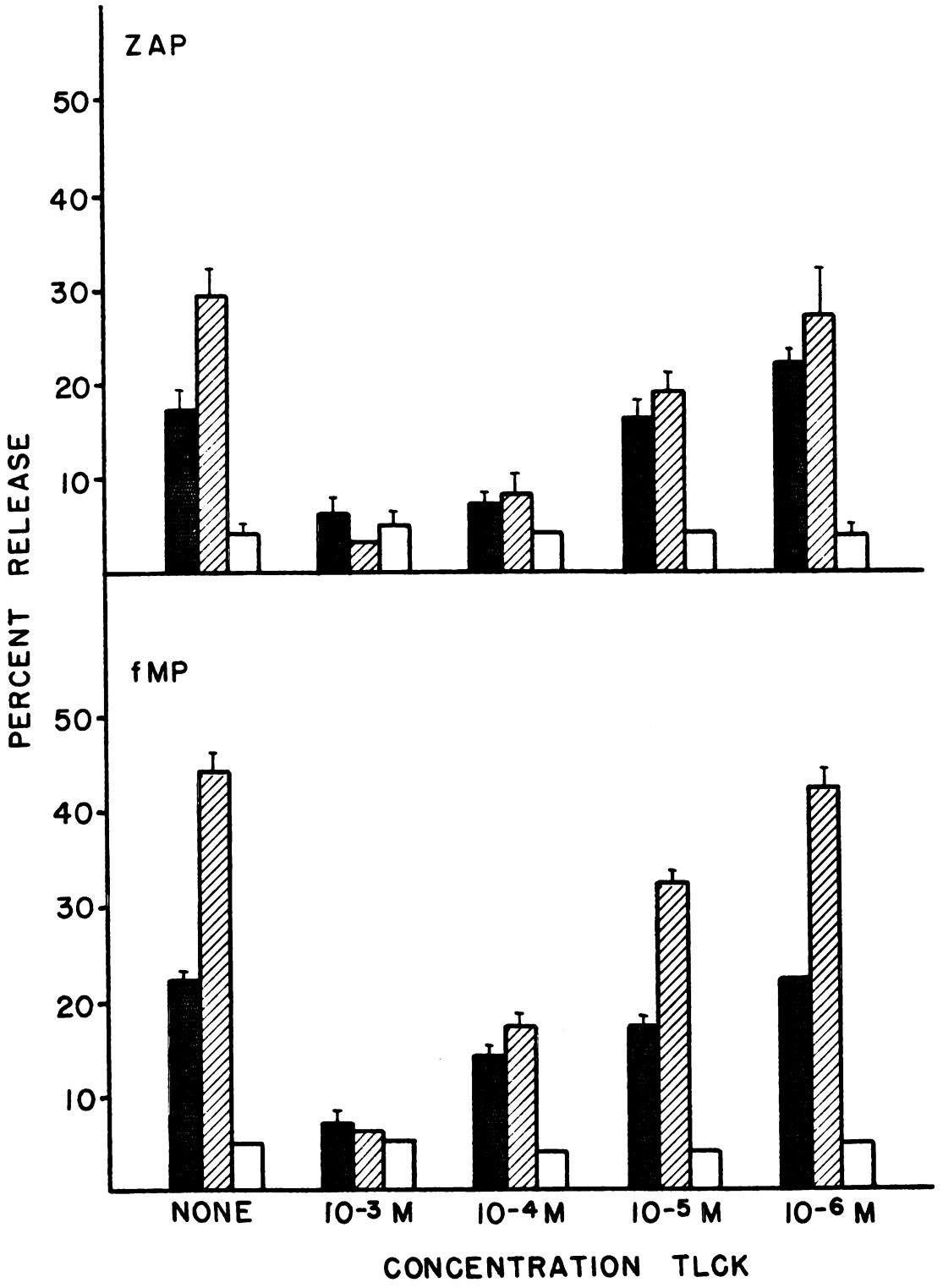

ZAP
50
40
30
20
10
PERCENT RELEASE
fMP
50
40
30
20
10
NONE
10-3 M
10-4 M
10-5 M
10-6 M
CONCENTRATION TLCK

Figure 1

Table 1. TLCK inhibition of lysosomal enzyme release[a]

Additions to cells	β-Glucuronidase	n	Lysozyme	n	LDH (n=7)
None	5.2±0.31[b]	6	6.3±0.42	13	5.9±0.38
ZAP only	24.4±1.3	6	25.3±0.61	14	6.1±0.90
ZAP+10^{-3}M TLCK	15.0±1.1[c]	6	11.9±1.5[c]	12	7.3±1.3
ZAP+10^{-4}M TLCK	24.7±1.1	6	23.3±1.1	12	6.5±1.6
ZAP+10^{-5}M TLCK	26.5±1.9	6	27.5±2.2	12	6.8±1.5
fMP only	43.1±0.25	4	50.0±2.5	11	8.2±0.80
fMP+10^{-3}M TLCK	22.0±0.21[d]	4	22.7±1.0[c]	12	7.7±1.1
fMP+10^{-4}M TLCK	37.7±0.73[e]	4	45.2±4.0	10	8.3±0.84
fMP+10^{-5}M TLCK	43.8±1.37	4	52.3±2.1	10	8.2±0.29

[a]ZAP release includes 10% ZAP + 5 μg/ml CB with 2 x 10^6 cells/ml incubated at 37°C for 30 minutes; fMP release includes 10^{-4}M fMP + 5 μg/ml CB + 1 mg/ml BSA with 2 x 10^6 cells/ml incubated at 37°C for 5 minutes.

[b]Results presented as % ± SEM of total enzyme content determined by treatment of 2 x 10^6 cells/ml with 0.2% Triton X-100 (β-Glucuronidase = 150 ± 3 μg phenolphthalein/ml, Lysozyme = 241 ± 9 units/ml, LDH = 399 ± 11 units/ml). Student's t-test p values for β-Glucuronidase and Lysozyme compared to release with stimulus only; p values for LDH compared to negative control; n = degrees of freedom. Only statistically significant p values annotated.

[c]$p<.02$.

[d]$p<.001$.

[e]$p<.01$.

Inhibition of Enzyme Release with the Chymotrypsin-Like Inhibitor TPCK

The same conditions for detecting inhibition of BG or lysozyme release were utilized for TPCK as described above for TLCK (i.e., in the absence or presence of releasing stimulus). Figure 2 shows the results when treating the cells with TPCK in the absence of a secretory stimulus. When using fMP, BG release was significantly depressed at 10^{-4} and 10^{-5}M TPCK and lysozyme release was significantly inhibited at these concentrations as well. No inhibition of lysozyme release was seen at 10^{-4}M when using ZAP-stimulated cells, as shown in Table 2. The same response was seen on BG release in data not presented here. The chymotryptic inhibitor and sulfonyl halide, PMSF, at 10^{-4}M did not affect lysozyme release by either ZAP or fMP under pretreatment conditions (also in Table 2).

The second protocol (i.e., inhibitor present with stimulus) was performed with TPCK and the results are shown in Table 3. When using this protocol, fMP-stimulated enzyme release was significantly depressed at 10^{-4}M TPCK only in the case of lysozyme. No effect was detected when assaying for primary granule release (BG). Analogous results were found with ZAP-stimulated cells. TPCK at 10^{-4}M inhibited lysozyme and not BG release.

LDH levels were again found to be low and unaffected by TPCK at 10^{-4}M or less.

Figure 2. TPCK pretreatment then fMP release. PMNs (2×10^6/ml) were preincubated with the inhibitor for 5 minutes at 37°C, washed once, then treated with 1 mg/ml BSA, 5 µg/ml CB, and 10^{-4}M fMP with incubation at 37°C for 5 minutes. Results presented as % ± SEM of total enzyme content determined by treatment of 2×10^6 cells/ml with 0.2% Triton X-100 (β-Glucuronidase = 139 ± 7 µg phenolphthalein/ml, Lysozyme = 165 ± 13 units/ml, LDH = 385 ± 27 units/ml). Graphic presentation of enzyme levels shown as solid bars (BG), hashed bars (LYS), and open bars (LDH). Negative controls for all 3 enzymes were not greater than 5.4 ± .85%. Student's t-test p values for BG (n=6) and LYS (n=6) were compared to tubes receiving no pretreatment, but subjected to fMP release. LDH p values (n=5) were compared to the negative control and none were significant. The inhibition on LYS at 10^{-4}M and 10^{-5}M TPCK was highly significant ($p<.005$). TPCK pretreatment effect on BG showed $p<.05$ at 10^{-4}M and $p<.025$ at 10^{-5}M.

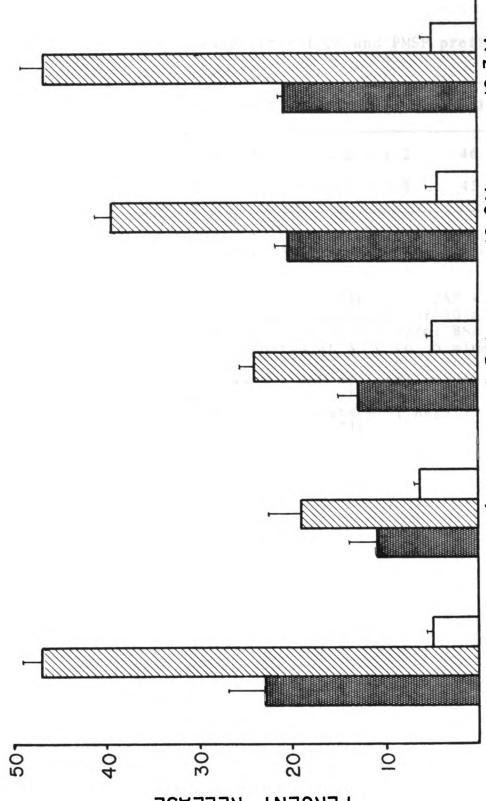

50
40
30
20
10
PERCENT RELEASE
NONE
10-4 M
10-5 M
10-6 M
10-7 M
CONCENTRATION TPCK

Figure 2

Table 2. Lysozyme release after TPCK and PMSF pretreatment[a]

Pretreatment	TPCK/ZAP[b] (n=3)	PMSF/ZAP (n=3)	PMSF/fMP (N=4)
None	26.0 ± 2.4[c]	32.5 ± 2.2	46.2 ± .33
10^{-4}M inhibitor	25.4 ± 1.9	32.3 ± 1.1	45.9 ± 1.3

[a]Pretreatment consists of 2 x 10^6 cells/ml incubated at 37°C for 5 minutes with 10^{-4}M inhibitor, washed once, then treated with stimulus.

[b]Inhibitor/Release Stimulus: ZAP = 10% ZAP + 5 µg/ml CB added to 2 x 10^6 cells/ml and incubated for 30 minutes at 37°C; fMP = 10^{-4}M fMP + 5 µg/ml CB + 1 mg/ml BSA added to 2 x 10^6 cells/ml and incubated at 37°C for 5 minutes.

[c]Values expressed as % ± SEM of total enzyme content determined by treatment of 2 x 10^6 cells/ml with 0.2% Triton X-100 (Lysozyme = 203 ± 8 units/ml); negative control values not greater than 6.0 ± .74%.

Table 3. TPCK inhibition of lysosomal enzyme release[a]

Additions to cells	β-Glucuronidase (n=6)	Lysozyme	n	LDH	n
None	4.9±0.25[b]	6.0±0.47	11	6.0±0.17	6
ZAP only	19.3±3.3	28.8±1.1	11	4.4±1.2	6
ZAP+10^{-4}M TPCK	15.4±4.1	23.0±1.5[c]	12	4.8±0.70	6
ZAP+10^{-5}M TPCK	16.5±3.5	29.3±0.87	10	4.8±0.29	6
ZAP+10^{-6}M TPCK	18.1±3.0	31.5±1.6	10	5.0±0.60	6
fMP only	26.8±2.5	47.4±1.8	12	8.8±0.79	10
fMP+10^{-4}M TPCK	21.6±3.7	34.1±2.7[d]	14	7.8±0.58	10
fMP+10^{-5}M TPCK	25.7±2.4	48.0±2.7	12	7.6±0.71	10
fMP+10^{-6}M TPCK	29.3±3.3	48.6±2.8	12	8.4±0.88	10

[a]ZAP release includes 10% ZAP + 5 μg/ml CB with 2 x 10^6 cells/ml incubated at 37°C for 30 minutes; fMP release includes 10^{-4}M fMP + 5 μg/ml CB + 1 mg/ml BSA with 2 x 10^6 cells/ml incubated at 37°C for 5 minutes.

[b]Results presented as % ± SEM of total enzyme content determined by treatment of 2 x 10^6 cells/ml with 0.2% Triton X-100 (β-Glucuronidase = 151 ± 4 μg phenolphthalein/ml; Lysozyme = 270 ± 7 units/ml; LDH = 466 ± 8 units/ml). Student's t-test p values for β-Glucuronidase and Lysozyme compared to release with stimulus alone; p values for LDH compared to negative control; n = degrees of freedom. Only statistically significant p values annotated.

[c]$p<.05$.

[d]$p<.02$.

Time Course of TLCK and TPCK Inhibition of Lysozyme Release with fMP

Cells were treated at varying time intervals (30 seconds to 10 minutes) with either 10^{-3}M TLCK or 10^{-4}M TPCK, washed, and treated with fMP as described in Methods. The extent of lysozyme secretion was subsequently assessed (data not shown). A marked inhibitory effect (a decrease to 15% from 45% release with untreated cells) was seen at 30 seconds, while maximum inhibition occurred at 2 minutes and remained unchanged at 10 minutes for both inhibitors.

Inhibition of Enzyme Release with BTEE

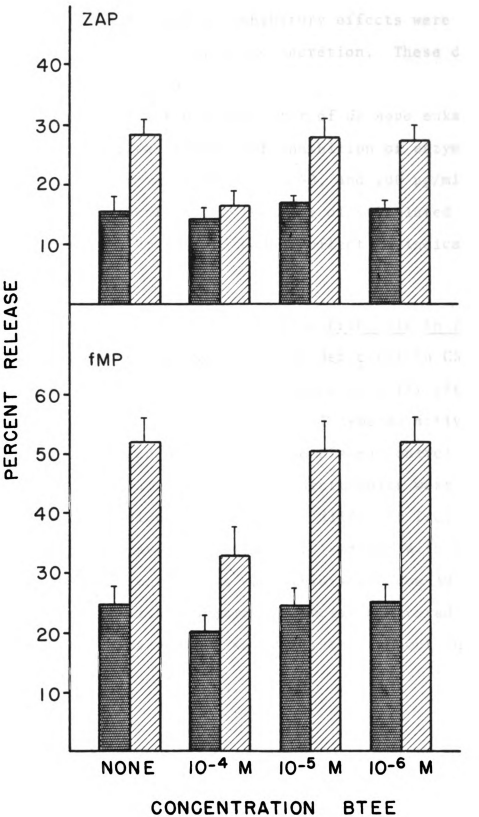
It seemed of interest to test for potential competitive inhibition of enzyme release by use of a synthetic substrate for chymotrypsin. Both protocols were used as already described. BTEE-treated neutrophils, followed by washing and stimulation by either ZAP or fMP, released both granule markers to the same extent as untreated control cells. However, as shown in Figure 3, when BTEE was maintained with ZAP or fMP during the period of stimulated release, significant inhibition of lysozyme was affected at 10^{-4}M. BG release with ZAP or fMP was unaffected under these conditions.

TAME, a substrate for trypsin, did not influence enzyme release under identical experimental conditions.

Lack of Inhibition of Enzyme Release with PMSF and Cycloheximide

Several attempts were made to detect inhibition of enzyme release with PMSF. Concentrations of 10^{-4}, 10^{-5},

Figure 3. BTEE inhibition of enzyme release. PMNs (2×10^6/ml) were incubated with BTEE and stimuli: (a) ZAP = 10% ZAP + 5 μg/ml CB for 30 minutes at 37°C or (B) fMP = 10^{-4}M fMP + 5 μg/ml CB + 1 mg/ml BSA for 5 minutes at 37°C. Values presented as % ± SEM of total enzyme content determined by treatment of 2×10^6 cells/ml with 0.2% Triton X-100 (β-Glucuronidase = 168 ± 13 μg phenolphthalein/ml and Lysozyme = 214 ± 12 units/ml). No effect seen on β-Glucuronidase release (solid bars) with either ZAP or fMP (n=4 for both); significant inhibition seen at 10^{-4}M BTEE on Lysozyme release (hashed bars) with ZAP (n=8) and fMP (n=6). Negative controls not greater than 6.7 ± .71%. No cytotoxicity observed under all conditions.


ZAP
40
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20
10
fMP
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40
30
20
10
PERCENT RELEASE
NONE
10-4 M
10-5 M
10-6 M
CONCENTRATION BTEE

Figure 3

and 10^{-6}M PMSF were utilized in both experimental protocols with ZAP and fMP and no inhibitory effects were detected for both BG and lysozyme secretion. These data are presented in Table 4.

Cycloheximide, a known inhibitor of *de novo* eukaryotic protein synthesis, was tested for inhibition of enzyme release at concentrations of 5, 10, 50, and 100 µg/ml. This agent was present during the time of stimulated enzyme secretion with ZAP or fMP and had no effect as indicated in Table 5.

Association of C5 with the Active Principle in ZAP

Normal human plasma was rendered deficient in C5 (see Methods) and tested for enzyme releasing activity after activation with zymosan. The results of zymosan activation were compared with an unabsorbed sham plasma control (see Methods) from the same individual. Neutrophils were used from the same individual as well as a different individual. In both cases, the average release of lysozyme with ZAP made from C5-deficient plasma was diminished from 39 to 15% when compared with unabsorbed, zymosan-activated controls. The release of BG was completely absent under these conditions.

Table 4. Effect of PMSF on enzyme release[a]

Addition to cells	β-Glucuronidase	n	Lysozyme	n	LDH(n=4)
None	6.1± .21[b]	3	7.9± .70	10	6.4±.69
ZAP only	20.2±1.9	4	29.3±1.9	10	7.3±.13
ZAP+10^{-4}M PMSF	20.7±1.2	4	26.7±3.4	10	7.3±.13
ZAP+10^{-5}M PMSF	20.0±1.5	4	34.0±2.1	9	5.9±.25
ZAP+10^{-6}M PMSF	21.9±2.0	4	35.6±1.6	9	5.6±.40
fMP only	34.0±3.2	2	51.7±3.7	10	7.4±.67
fMP+10^{-4}M PMSF	31.7±4.2	2	44.6±5.0	10	7.3±.40
fMP+10^{-5}M PMSF	32.6±4.0	2	54.2±5.2	9	8.1±.60
fMP+10^{-6}M PMSF	32.3±4.6	2	54.7±5.2	9	7.8±.81

[a]Inhibitor present with stimulus: ZAP = 10% ZAP + 5 μg/ml CB with 2 x 10^6 cells/ml incubated at 37°C for 30 minutes; fMP = 10^{-4} fMP + 5 μg/ml CB + 1 mg/ml BSA with 2 x 10^6 cells/ml incubated at 37°C for 5 minutes.

[b]Values presented at % ± SEM of total enzyme content determined by treatment of 2 x 10^6 cells/ml with 0.2% Triton X-100 (β-Glucuronidase = 135 ± 9 μg phenolphthalein/ml, Lysozyme = 254 ± 22 units/ml, LDH = 450 ± 10 units/ml). Student's t-test p values not significant for data shown (BG and Lysozyme values compared to release tubes alone, LDH values compared to negative control), n = degrees of freedom.

Table 5. Effect of cycloheximide on lysozyme release[a]

Inhibitor Concentration (µg/ml)	ZAP (n=3)	FMP (n=3)
0	39.4 ± 1.4[b]	57.1 ± 2.7
5	38.7 ± .11	56.8 ± .07
10	39.4 ± 1.4	58.5 ± 4.5
50	40.0 ± .99	57.1 ± 2.7
100	37.8 ± .78	55.9 ± 2.6

[a]Inhibitor added with stimulus: ZAP = 10% ZAP + 5 µg/ml CB added to 2 x 10^6 cells/ml incubated at 37°C for 30 minutes; FMP = 10^{-4}M fMP + 5 µg/ml CB + 1 mg/ml BSA added to 2 x 10^6 cells/ml incubated at 37°C for 5 minutes.

[b]Values presented as % ± SEM of total enzyme content determined by treatment of 2 x 10^6 cells/ml with 0.2% Triton X-100 (lysozyme = 206 ± 10 units/ml); negative control = 7.9 ± .81%.

DISCUSSION

Cell-related esterase activity has been demonstrated in PMN chemotaxis, phagocytosis, and lysosomal degranulation by the use of organophosphate esters. For neutrophil chemotaxis, Ward and Becker (67) defined two forms of esterase inhibition based upon the inhibition profile of organophosphorus analogues. The cell-dependent inhibition occurred in the absence of a chemotactic stimulus indicating the presence of an activated esterase. The chemotactic factor-dependent inhibition of chemotaxis was concluded to be the result of an activatable esterase. The two activities were not caused by identical proteases since the inhibition profiles were different.

These studies prompted us to study the effect of protease inhibitors in order to discern the potential role of serine esterases and proteases in lysosomal enzyme release. Because of their unique specificities and identical inactivating mechanisms, the chloromethylketones provide excellent tools for the analysis of tryptic (TLCK) and chymotryptic (TPCK) activity that may relate to cell function. Our approach was to investigate lysosomal enzyme release by exposing human neutrophils to these and related inhibitors in the presence and absence of releasing stimuli.

Table 6 summarizes in a qualitative fashion our findings as they relate to primary and secondary granule secretion induced by ZAP and fMP. We have categorized the altered neutrophil response in terms of either an activatable (i.e., inhibitor with stimulus) or an activated (i.e., inhibitor only) esterase. The evidence clearly suggests that both trypsin-like and chymotrypsin-like enzymes are associated with lysosomal degranulation. Use of the tryptic inactivator TLCK, either as a cell-dependent or stimulus-dependent inhibitor, impeded enzyme release regardless of the stimulus used to induce degranulation. This indicates to us that an activated tryptic enzyme is associated with enzyme release. Although it seems clear that an activated esterase is functional, our experimental conditions do not conclusively demonstrate an activatable esterase; however, we have not ruled out its presence. The tryptic competitive inhibitor TAME did not help us in this regard since it did not affect enzyme release under any circumstances.

The chymotryptic response to lysosomal secretion was more discriminating. TPCK did not inhibit C5-related (ZAP) release in the absence of stimulus; however, primary and secondary degranulation with fMP were blocked. This suggests the presence of an activated chymotryptic protease which responds differently to chemotactic stimuli. We can speculate that differences in cell-binding sites for ZAP (C5) and fMP are involved in the observed effect. A clear

Table 6. Summary of the inhibitory effects on lysosomal enzyme release[a]

	ZAP (1°)	ZAP (2°)	fMP (1°)	fMP (2°)
A. Inhibitor + Stimulus:				
TLCK	+	+	+	+
TPCK	-	+	-	+
PMSF	-	-	-	-
BTEE	-	+	-	+
TAME	-	-	-	-
B. Inhibitor Pretreatment:				
TLCK	+	+	+	+
TPCK	-	-	+	+
PMSF	-	-	-	-
BTEE	ND[b]	ND	-	-

[a]Significant inhibition is expressed with positive (+) signs for the conditions given. Stimuli, ZAP or fMP, are followed by ( ) indicating primary or secondary granule response based upon BG and LYS release data.

[b]ND = not done.

correlation can be seen between chymotryptic activity and secondary degranulation when inhibitor and stimulus are present simultaneously, since both TPCK and BTEE had the same inhibitory effect. Lysozyme release was effectively blocked while primary granule secretion was unaffected. This suggests that an activatable chymotryptic enzyme selectively influences secondary neutrophil chemotactic responsiveness and protease inhibitors. The chloromethyl-ketones inhibit chemotaxis with an activated chymotryptic esterase appearing to have the predominant role (2,25). Our findings are in agreement with this suggesting that chemotaxis and lysosomal enzyme release require similar or identical esterases, but more specifically a probable correlation may exist between secondary lysosomal degranulation and PMN chemotaxis.

Becker et al. (10) showed that complement-derived factors such as C5a are capable of inducing lysosomal enzyme release. This reinforced an earlier study done by Goldstein et al. (27) implicating C5 activity with the ability of zymosan-activated serum to cause selective degranulation. Through immunoadsorbence of C5 from plasma, we have demonstrated that the enzyme releasing ability of ZAP is almost entirely due to C5. Secondary granule release was reduced 80% with C5 removal while primary degranulation was completely absent.

The use of synthetic protease inhibitors definitely helps to characterize esterase involvement; however, usage

of naturally-occurring alpha-globulin inhibitors provides a relevant basis for studying protease-related cell activity. Recently, we have investigated fMP-induced lysosomal secretion from neutrophils previously incubated with highly purified preparations of human C1 inactivator (C1INA). The concentrations of C1INA incorporated were slightly below (5 units/ml) and above (10 units/ml) the normal physiological level of 7 units/ml. At the higher concentration, purified C1INA significantly suppressed secondary granule secretion but did not affect primary release. The lower level of C1INA did not impede the release of either marker enzyme. This discrimination toward secondary granule inhibition correlates with the activatable chymotryptic esterase data previously discussed. It has been shown that human neutrophils in the presence of C1INA demonstrate an enhanced chemotactic response to both ZAP (59) and fMP (44). The critical experiment yet to be accomplished involves specific removal of C1INA from the highly purified preparation by use of a monospecific immune adsorption. This will hopefully establish that C1INA is the active principle in the preparation that alters enzyme release. If successful, the experiment will implicate C1INA as a biological control of neutrophil degranulation. Of equal importance, therefore, is the usage of other alpha-globulin inhibitors to determine how they will affect degranulation from neutrophils.

SUMMARY AND CONCLUSIONS

This study was designed to focus on the cell-related esterase activity associated with neutrophil lysosomal enzyme release. Through the use of chloromethylketones and certain model substrates, the participation of both tryptic and chymotryptic enzymes was implicated. The trypsin-like response was inhibitable with TLCK regardless of the inactivating protocol or releasing stimulus. The chymotryptic activity responding to TPCK showed inhibition of fMP-stimulated and not ZAP-induced release in the absence of stimulus. When the inhibitor and releasing agent were together, only secondary degranulation was affected. The chymotryptic substrate, BTEE, competitively suppressed enzyme release, whereas the tryptic substrate, TAME, was ineffective. Preliminary experiments with highly purified preparations of C1INA strongly implicate this and perhaps other alpha-globulin plasma inhibitors in the natural control of this important neutrophil function.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Aldridge, D., Armstrong, J., Speake, R., and Turner, W. The cytochalasins, a new class of biologically active mould metabolites. Chem. Comm. 1:26-27, 1967.

2. Aswanikumar, S., Schiffmann, E., Corcoran, B., and Wahl, S. Role of a peptidase in phagocyte chemotaxis. Proc. Nat'l. Acad. Sci. 73, no. 7:2439-2442, 1976.

3. Aswanikumar, S., Corcoran, B., Schiffmann, E., Day, A., Freer, R., Showell, H., Becker, E., and Pert, C. Demonstration of a receptor on rabbit neutrophils for chemotactic peptides. Biochem. Biophys. Res. Commun. 74, no. 2:810-817, 1977.

4. Axelsen, N. *Quantitative Immunoelectrophoresis: New Developments and Applications.* Oslo: Universitets forlaget, pp. 39-50, 1975.

5. Baggiolini, M., Hirsch, J., and DeDuve, C. Resolution of granules from rabbit heterophil leukocytes into distinct populations by zonal sedimentation. J. Cell Biol. 40:529-537, 1969.

6. Bainton, D., and Farquhar, M. Differences in enzyme content of azurophil and specific granules of polymorphonuclear leukocytes. II. Cytochemistry and electron microscopy of bone marrow cells. J. Cell Biol. 39:299-317, 1968.

7. Bainton, D. Sequential discharge of polymorphonuclear leukocyte granules during phagocytosis of microorganisms. J. Cell Biol. 47(2, Pt. 2):11a (abstr), 1970.

8. Becker, E. The relationship of the chemotactic behavior of complement-derived factors C3a, C5a, and C567 and a bacterial chemotactic factor to their ability to activate the proesterase I of rabbit polymorphonuclear leukocytes. J. Exp. Med. 135:376-387, 1972.

9. Becker, E., Davis, A., Estensen, R., and Quie, P. Cytochalasin B. IV. Inhibition and stimulation of chemotaxis of rabbit and human polymorphonuclear leukocytes. J. Immunol. 108:396-402, 1972.

10. Becker, E., Showell, H., Henson, P., and Hsu, L. The ability of chemotactic factors to induce lysosomal enzyme release. I. The characteristics of the release, the importance of surfaces and the relation of enzyme release to chemotactic responsiveness. J. Immunol. 112:2047-2054, 1974.

11. Becker, E., and Showell, H. The ability of chemotactic factors to induce lysosomal enzyme release. II. The mechanism of release. J. Immunol. 112:2055-2062, 1974.

12. Becker, E. Enzyme activation and the mechanism of polymorphonuclear leukocyte chemotaxis. In *The Phagocyte Cell in Host Resistance*. New York: Raven Press, ed. by Bellanti and Davies, pp. 1-11, 1975.

12. Becker, E. Some interrelations of neutrophil chemotaxis, lysosomal enzyme secretion and phagocytosis as revealed by synthetic peptides. Am. J. Path. 85: 385-394, 1976.

14. Bergmeyer, H., Bernt, E., and Hess, B. Lactic dehydrogenase. In *Methods of Enzymatic Analysis*. New York and London: Academic Press, ed. by Bergmeyer, pp. 736-740, 1963.

15. Borel, J. and Stahlein, H. Effects of cytochalasin B on chemotaxis and immune reactions. Experientia 28: 745 (abstr), 1972.

16. Canarozzi, N., and Malawista, S. Phagocytosis of human blood leukocytes measured by uptake of ^{131}I-labelled human serum albumin: inhibitory and stimulatory effects of cytochalasin B. Yale J. Biol. Med. 46:177-189, 1973.

17. Carter, S. Effects of cytochalasins on mammalian cells. Nature 213:261-266, 1967.

18. Chou, I., Black, P., and Roblin, R. Nonselective inhibition of transformed cell growth by a protease inhibitor. Proc. Nat'l. Acad. Sci. 71:1748-1752, 1976.

19. Cochrane, C. Immunologic tissue injury mediated by neutrophilic leukocytes. Adv. Immunol. 9:97-162, 1968.

20. Davies, P., Allison, A., and Haskwell, A. Selective release of lysosomal hydrolases from phagocytic cells by cytochalasin B. Biochem. J. 134:33-41, 1973.

21. Davies, A., Estenson, R., and Quie, P. Cytochalasin B. III. Inhibition of human polymorphonuclear leukocyte phagocytosis. Proc. Soc. Exp. Biol. Med. 137:161-164, 1971.

22. DeDuve, C., and Wattiaux, R. Functions of lysosomes. Ann. Rev. Physiol. 28:435-443, 1966.

23. Fishman, W. Beta-Glucuronidase. In *Methods of Enzymatic Analysis.* New York: Academic Press, ed. by Bergmeyer, pp. 869-874, 1963.

24. Gallin, J., Durocher, J., and Kaplan, A. Interaction of leukocyte chemotactic factors with the cell surface. I. Chemotactic factor-induced changes in human granulocyte surface charge. J. Clin. Invest. 55:967-974, 1975.

25. Goetzel, E. Modulation of neutrophil polymorphonuclear leukocyte migration by human plasma alpha-globulin inhibitors and synthetic esterase inhibitors. Immunology 29:163-174, 1975.

26. Gold, A. Sulfonylation with sulfonyl halides. In *Methods of Enzymology, Vol. XI. Enzyme Structure.* New York and London: Academic Press, ed. by Hirs, pp. 706-711, 1967.

27. Goldstein, I., Brai, M., Osler, A., and Weissmann, G. Lysosomal enzyme release from human leukocytes. Mediation of the alternate pathway of complement activation. J. Immunol. 111:33-37, 1973.

28. Goldstein, I., Hoffstein, S., Gallin, J., and Weissmann, G. Mechanisms of lysosomal enzyme release from human leukocytes: Microtubule assembly and membrane fusion induced by a component of complement. Proc. Nat'l. Acad. Sci. 70:2916-2920, 1973.

29. Grinnell, F. Cell attachment to a substratum and cell surface proteases. Arch. Biochem. Biophys. 169:474-482, 1975.

30. Hawkins, D. Biopolymer membrane: A model system for the study of the neutrophilic leukocytic response to immune complexes. J. Immunol. 107, no. 2:344-352, 1971.

31. Hawkins, D. Inhibition of neutrophil lysosomal release. Fed. Proc. Immunol. 31:748 (abstr), 1972.

32. Hawkins, D. Neutrophilic leukocytes in immunologic reactions: Evidence for selective release of lysosomal constituents. J. Immunol. 108:310-317, 1972.

33. Heimberger, N. Proteinase inhibitors of human plasma - their properties and control functions. In *Proteases and Biological Control.* Cold Spring Harbor Laboratory, ed. by Reich, Rifkin and Shaw., pp. 367-386, 1975.

34. Henson, P. Interaction of cells with immune complexes: Adherence, release of constituents and tissue injury. J. Exp. Med. 134(3, Pt. 2):114-135, 1971.

35. Henson, P., Johnson, H., and Spiegelberg, H. The release of granule enzymes from human neutrophils stimulated by aggregated immunoglobulins of different classes and subclasses. J. Immunol. 109:1182-1192, 1972.

36. Henson, P. Mechanisms of release of granule enzymes from human neutrophils phagocytosing aggregated immunoglobulin. Arth. Rheum. 16:208-216, 1973.

37. Henson, P. Mechanisms of release of granule enzymes from human neutrophils by cytochalasin B. J. Immunol. 110:290-293, 1973.

38. Henson, P. Mechanisms of activation and secretion by platelets and neutrophils. Progr. in Immunology II, 2:95-105, 1974.

39. Hoffstein, S., Soberman, R., Goldstein, I., and Weissmann, G. Concanavalin A induces microtubule assembly and specific granule discharge in human polymorphonuclear leukocytes. J. Cell Biol. 68: 781-787, 1976.

40. Hoffstein, S., Goldstein, I., and Weissmann, G. Role of microtubule assembly in lysosomal enzyme secretion from human polymorphonuclear leukocytes. A reevaluation. J. Cell Biol. 73:242-256, 1977.

41. Hsu, L., and Becker, E. Volume changes induced in rabbit polymorphonuclear leukocytes by chemotactic factor and cytochalasin B. Am. J. Path. 81:1-14, 1975.

42. Koza, E., Wright, T., and Becker, E. Lysosomal enzyme secretion and volume contraction induced in neutrophils by cytochalasin B, chemotactic factor, and A23187. Proc. Soc. Exp. Biol. Med. 149:476-479, 1975.

43. Lefell, M., and Spitznagel, J. Intracellular and extracellular degranulation of human polymorphonuclear azurophil and specific granules by immune complexes. Infect. Immun. 10:1241-1248, 1974.

44. Liu, D. Human C1INH effects on chemotactic responsiveness of human neutrophils. Dissertation for the degree of PhD, Michigan State University, 1976.

45. Malawista, S., and Bensch, K. Human polymorphonuclear leukocytes: Demonstration of microtubules and effect of colchicine. Science 156:521-526, 1967.

46. Malawista, S., Gee, J., and Bensch, K. Cytochalasin B reversibly inhibits phagocytosis: Functional metabolic and ultrastructural effects in human blood leukocytes and rabbit alveolar macrophages. Yale J. Biol. Med. 44:286-300, 1971.

47. Musson, R., and Becker, E. The inhibitory effect of chemotactic factors on erythrophagocytosis by human neutrophils. J. Immunol. 117, no. 2:433-439, 1976.

48. Naccache, P., Showell, H., Becker, E., and Sha'afi, R. Changes in ionic movement across rabbit polymorphonuclear leukocyte membranes during lysosomal enzyme release. Possible ionic basis for lysosomal enzyme release. J. Cell Biol. 75:635-649, 1977.

49. Nilsson, U., and Muller-Eberhard, H. Isolation of β_1F-globulin from human serum and its characterization of the fifth component of complement. J. Exp. Med. 122:277-298, 1965.

50. O'Dea, R., Viveros, O., Axelrod, J., Aswanikumar, S., Schiffmann, E., and Coccoran, B. Rapid stimulation of protein carboxymethylation in leukocytes by a chemotactic peptide. Nature 272:462-464, 1978.

51. Pearlman, D., Ward, P., and Becker, E. The requirement of serine esterase in complement-dependent erythrophagocytosis. Nomen. Comp. Bull. WHO 39:745-764, 1968.

52. Roos, D., Homan-Muller, J., and Weening, R. Effect of cytochalasin B on oxidative metabolism of human peripheral blood granulocytes. Biochem. Biophys. Res. Commun. 68, no. 1:43-50, 1976.

53. Scheidegger, J. A micromethod of immunoelectrophoresis. Int. Arch. Allergy Appl. Immunol. 7:103-110, 1955.

54. Schiffmann, E., Corcoran, B., and Wahl, S. N-formylmethionyl peptides as chemoattractants for leukocytes. Proc. Nat'l. Acad. Sci. 72:1059-1062, 1975.

55. Showell, H., Freer, R., Zigmond, S., Schiffmann, E., Aswanikumar, S., Corcoran, B., and Becker, E. The structure-activity relations of synthetic peptides as chemotactic factors and inducers of lysosomal enzyme secretion for neutrophils. J. Exp. Med. 143:1154-1169, 1976.

56. Shugar, D. Measurement of lysozyme activity and ultraviolet inactivation of lysozyme. Biochim. Biophys. Acta 8:302-309, 1952.

57. Skosey, J., Damgaard, E., Chow, D., and Sorensen, L. Modification of zymosan-induced release of lysosomal enzymes from human polymorphonuclear leukocytes by cytochalasin B. J. Cell Biol. 62:625-634, 1974.

58. Smith, W., Hollers, J., Dupree, E., Goldman, A., and Lord, R. A serum inhibitor of leukocytes in a child with recurrent infections. J. Lab. Clin. Med. 79: 878-885, 1972.

59. Smith, W., Hollers, J., Bing, D., and Patrick, R. Effects of human C1 inhibitor on complement-mediated human leukocyte chemotaxis. J. Immunol. 114 (1, Pt. 1):216-220, 1975.

60. Steinbuch, M., and Audran, R. The isolation of IgG from mammalian sera with the aid of caprylic acid. Arch. Biochem. Biophys. 134:279-284, 1969.

61. Taubman, S., Goldschmidt, P., and Lepow, I. Effects of lysosomal enzymes from human leukocytes on complement components. Fed. Proc. Immunol. 29:434 (abstr), 1970.

62. Taubman, S., and Lepow, I. Cleavage of human $C1_S$ by human lysosomal enzymes, plasmin, and trypsin. Immunochem. 10:651-656, 1971.

63. Taylor, J., Crawford, I., and Hughli, T. Limited degradation of the third component (C3) of human complement by human leukocyte elastase (HLE): Partial characterization of C3 fragments. Biochemistry 16, No. 15:3390-3396, 1977.

64. Tou, J., and Stjernholm, R. Cytochalasin B: Effect on phospholipid metabolism and lysosomal enzyme release by leukocytes. Biochim. Biophys. Acta 392: 1-11, 1975.

65. Tsung, R., Kregeles, S., Showell, H., and Becker, E. Isolation of an N-acetyl-DL-phenylalanine-B-naphthyl esterase from rabbit peritoneal polymorphonuclear leukocytes. Biochim. Biophys. Acta 403:98-105, 1975.

66. Tsung, R., Kregeles, S., and Becker, E. Some differences in intracellular distribution and properties of the chymotrypsin-like esterase activity of human and rabbit neutrophils. Biochim. Biophys. Acta 499:212-226, 1977.

67. Ward, P., and Becker, E. Mechanisms of the inhibition of chemotaxis by phosphonate esters. J. Exp. Med. 125:1001-1020, 1967.

68. Ward, P., and Hill, J. C5 chemotactic fragments produced by an enzyme in lysosomal granules of neutrophils. J. Immunol. 104, No. 3:535-543, 1970.

69. Weissmann, G., Zurier, R., Spieler, P., and Goldstein, I. Mechanisms of lysosomal enzyme release from leukocytes exposed to immune complexes and other particles. J. Exp. Med. 134:149-165, 1971.

70. Weissmann, G., Zurier, R., and Hoffstein, S. Leukocytic proteases and the immunologic release of lysosomal enzymes. Am. J. Path. 68, No. 3:539-564, 1972.

71. Welsh, I. and Spitznagel, J. Distribution of lysosomal enzymes, cationic proteins, and bactericidal substances in subcellular fractions of human polymorphonuclear leukocytes. Infect. and Immun. 4, No. 2:97-102, 1971.

72. Williams, L., Snyderman, R., Pike, M., and Lefkourtz, R. Specific receptor sites for chemotactic peptides on human polymorphonuclear lejkocytes. Proc. Nat'l. Acad. Sci. 74:1204-1208, 1977.

73. Wright, D., and Gallin, J. Inactivation of the chemotactic molecule C5a by products contained in human polymorphonuclear leukocytes (PMNs) and released during phagocytosis. Fed. Proc. 34:1019 (abstr), 1975.

74. Wright, D., and Gallin, J. Mechanisms for modulation of the inflammatory response: Generation and inactivation of C5a by products stored in the granules of human neutrophils. In *Leukocyte Chemotaxis*. New York: Raven Press, ed. by Gallin and Quie, pp. 211-228, 1978.

75. Zigmond, S., and Hirsch, J. Effects of cytochalasin B on polymorphonuclear leukocyte locomotion, phagocytosis and glycolysis. Exp. Cell Res. 73:383-393, 1972.

76. Zurier, R., Hoffstein, S., and Weissman, G. Cytochalasin B: Effect on lysosomal enzyme release from human leukocytes. Proc. Nat'l. Acad. Sci. 70:844-848, 1973.

77. Zurier, R., Weissmann, G., Hoffstein, S., Kammerman, S., and Tai, H. Mechanisms of lysosomal enzyme release from human leukocytes. II. Effects of cAMP and cGMP, autonomic agonists and agents which affect microtubule function. J. Clin. Invest. 53:297-303, 1974.

VITA

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