The Role of Lysosomal Elastase in the Digestion of *Escherichia coli* Proteins by Human Polymorphonuclear Leukocytes

EXPERIMENTS WITH LIVING LEUKOCYTES

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ABSTRACT Human polymorphonuclear leukocyte (PMN) elastase has been implicated in various pathological conditions. However, its physiological role remains undefined. One possible function of this enzyme may be digestion of bacterial proteins after phagocytosis. To test this hypothesis, we prepared *Escherichia coli* labeled with [^{3}H]arginine and treated these bacteria with a lipid-soluble, active-site-directed chloromethyl ketone inactivator of pancreatic and granulocyte elastases (carbobenzoxy-L-glycyl-L-leucyl-L-alanine chloromethyl ketone, dissolved in methanol). Control bacteria were treated with methanol alone. When *E. coli* pretreated with the inactivator were incubated with solutions of porcine pancreatic elastase or with PMN granule extract, release of trichloroacetic acid-soluble radioactivity was significantly lower than in the control *E. coli*. Similar results were obtained when treated and control *E. coli* were fed to viable human PMN. In contrast, release of trichloroacetic acid-soluble radioactivity from *E. coli* containing [^{3}H]thymidine was not affected by pretreatment of bacteria with elastase inactivator before feeding them to PMN, suggesting that phagocytosis of *E. coli* had not been inhibited by the chloromethyl ketone. When treated and control bacteria were fed to PMN, no significant difference was observed in the activity of lysosomal β-glucuronidase recovered from post-granule supernatant fractions of homogenized leukocytes, suggesting that lysosomal degranulation had not been suppressed by the inactivator. However, elastase activity of the same fractions was depressed if the leukocytes had phagocytized chloromethyl ketone-treated *E. coli*, suggesting that inhibition of PMN elastase had occurred. We conclude that PMN elastase participates in digestion of *E. coli* proteins by human PMN.

INTRODUCTION

Human granulocyte elastase, because of its neutral pH optimum and broad proteolytic activity, has been implicated in several disease processes, including rheumatoid arthritis (1, 2) and pulmonary emphysema (3). However, the physiological function of the elastase remains unknown. One possibility may be the digestion of bacterial protein after phagocytosis of microbes by polymorphonuclear leukocytes (PMN).[1] This view is supported by two previous observations from this laboratory (4, 5). In the first (4), evidence was presented showing that PMN elastase can lyse cell walls of autoclaved bacteria, when the latter are species whose peptidoglycan cross-linkages contain amino acid sequences similar to those present in tropoelastin or in cross-linkages of elastin fibers. In the second (5), a cell-free

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[1] *Abbreviations used in this paper:* AcAla$_3$NA, *N*-acetyl-L-alanyl-L-alanyl-L-alanine-*p*-nitroanilide; CBZ-Gly-Leu-Ala-CMK, carbobenzoxy-L-glycyl-L-leucyl-L-alanine chloromethyl ketone; CBZ-Gly-Leu-Phe-CMK, carbobenzoxy-L-glycyl-L-leucyl-L-phenylalanine chloromethyl ketone; HBSSG, Hanks' balanced salt solution with 0.15% glucose; PMN, polymorphonuclear leukocytes; TCA, trichloroacetic acid.

system was used to show that PMN elastase is a major contributor to the digestion of cytoplasmic *Escherichia coli* proteins by human leukocyte granular extracts in vitro. It remained to be shown, however, that elastase has a similar function within living leukocytes.

We now present results of experiments with living PMN and intact *E. coli* in which the role of leukocyte elastase in protein digestion of phagocytosed bacteria has been demonstrated. *E. coli* labeled with radioactive amino acids were pretreated either with methanol (control) or with carbobenzoxy-L-glycyl-L-leucyl-L-alanine chloromethyl ketone (CBZ-Gly-Leu-Ala-CMK) dissolved in methanol. The latter agent is a lipid-soluble, specific, active-site-directed, irreversible inactivator of pancreatic and leukocyte elastases (6). Our results will show that treatment of *E. coli* with this agent did not interfere with subsequent phagocytosis of the treated microorganisms by PMN, nor with leukocyte degranulation after phagocytosis. The latter was evaluated by monitoring the activity of β-glucuronidase, a PMN lysosomal enzyme (7), in post-granule supernatant fractions of leukocyte homogenates. Elastase activity of these homogenate fractions, as expected, was decreased by inactivator-treated bacteria. In conjunction with this, release of trichloroacetic acid (TCA)-soluble radioactivity was also partly suppressed by the inactivator. This inhibition effect was restricted to protein degradation, since release of TCA-soluble isotope from labeled nucleic acids was unaffected by the same chloromethyl ketone. These results confirm previous observations from this laboratory on the role of PMN elastase in the digestion of bacterial proteins, and extend our observations to viable, functioning leukocytes.

METHODS

Preparation of leukocytes

60 ml of venous blood was freshly drawn from normal human donors (males and females). 55 ml were anticoagulated by addition of 9 ml of acid citrate dextrose solution, formula A (8). 5 ml blood was allowed to clot at room temperature for 30 min. After clotting, serum was collected by centrifugation at 1,405 *g* for 10 min and reserved at 0°C until subsequent addition to incubation mixtures. Total leukocytes were counted in samples of the blood-acid citrate dextrose mixture with a hemocytometer. Typically, $2\text{–}3 \times 10^6$ leukocytes were present in 55 ml blood. The blood-acid citrate dextrose mixture was then mixed with 133 ml of 3% dextran (Dextran T-500, Pharmacia Fine Chemicals, Uppsala, Sweden) in physiological saline, and erythrocytes were allowed to settle for 40 min at 4°C. Leukocytes were obtained by centrifugation of the dextran supernate in siliconized bottles at 160 *g* for 20 min. Remaining erythrocytes were then lysed by hypotonic shock in 0.21% NaCl for 30 s. Lysis was terminated by adding KCl to a final concentration of 0.15 M. After the cell suspension was centrifuged at 180 *g* for 10 min, the leukocyte pellet was resuspended in normal saline and the erythrocyte lysis procedure was repeated. The leukocyte pellet was washed in Hanks' balanced salt solution containing 0.15% glucose (HBSSG), and the final cell pellet was resuspended in HBSSG to give a leukocyte concentration of 5×10^7 cells/ml. Typically, PMN leukocytes constituted 90% of the cells, as determined by differential counts of cell smears stained with Wright's stain. Leukocyte viability was tested by trypan blue dye exclusion at the beginning and end of the experiments described below. Over 99% of the PMN excluded trypan blue throughout the experimental period.

Preparation of bacteria

E. coli (strain K-MX72 T2) was obtained from Dr. M. Inouye (Department of Biochemistry, Stony Brook University). *E. coli* labeled with [^{3}H]arginine were grown as previously described (5). Doubly labeled *E. coli* were grown similarly, as previously described (5), with the following modification. When the organisms had entered logarithmic growth, 5 μCi [^{14}C]arginine and 50 μCi [^{3}H]thymidine were added per 100 ml of culture medium.

Treatment of bacteria with enzyme inactivators

Chloromethyl ketone inactivators of proteases, CBZ-Gly-Leu-Ala-CMK, and carbobenzoxy-L-glycyl-L-leucyl-L-phenylalanine chloromethyl ketone (CBZ-Gly-Leu-Phe-CMK), were synthesized and provided by Drs. J. Powers and P. M. Tuhy (Georgia Institute of Technology, Atlanta, Ga.). CBZ-Gly-Leu-Ala-CMK is a highly specific, active-site-directed inactivator of porcine pancreatic elastase (EC 3.4.4.7) and human granulocyte elastase (6). CBZ-Gly-Leu-Phe-CMK is a highly specific, active-site-directed inactivator of bovine α-chymotrypsin (EC 3.4.4.5) (6), and it also inactivates human granulocyte chymotrypsin-like enzyme.[2] Granulocyte chymotrypsin is a second, serine-dependent protease present in human PMN lysosomes (9). CBZ-Gly-Leu-Ala-CMK and CBZ-Gly-Leu-Phe-CMK were each dissolved in 75 μl methanol to give a final concentration of 0.4 M. *E. coli* (2×10^{11} organisms) were suspended in 675 μl distilled water and the inactivator-methanol solutions or 75 μl of methanol alone (control) were added to the *E. coli* suspensions. The final concentrations of inactivators and methanol were 40 mM and 10%, respectively. The incubation mixtures were shaken for 5 h at 125 rpm in a 37°C water bath (Aquatherm, New Brunswick Scientific Co., Inc., New Brunswick, N. J.). After incubation, 20 ml of 0.01 M sodium phosphate-buffered saline, pH 7.0, were added to the incubation mixtures to dilute the methanol and precipitate any inactivator that had not become associated with bacterial particles. The bacteria were then passed through a 6 μm Millipore filter (Millipore Corp., Bedford, Mass.) to remove any aggregates of excess, precipitated inactivator that might have formed. The filtrate, containing bacteria and associated inactivator, was diluted to an appropriate volume with HBSSG and stored in 1.0-ml portions at −20°C until used. Preliminary trials with ^{3}H-labeled chloromethyl ketones and unlabeled bacteria showed that a small but measurable fraction (3.0%) of starting radioactivity remained associated with bacteria after the above treatment. This associated label resisted repeated washing in aqueous buffers, but could be subsequently dissociated from the bacteria by washing them in methanol.

[2] Blondin, J., and Janoff, A. Unpublished observations.

Incubation mixtures

Pilot Experiments with Soluble Enzymes

E. coli labeled with [^{3}H]arginine, as described above, and treated with CBZ-Gly-Leu-Ala-CMK, CBZ-Gly-Leu-Phe-CMK, or methanol alone were resuspended in 1.2 ml of 0.05 M Tris-HCl buffer (pH 7.6 at 37°C) containing 0.1 M NaCl and 1 mM $CaCl_2$. Bacterial suspensions were adjusted to give an optical density of 0.5 at 520 nm. Porcine pancreatic elastase (EC 3.4.4.7) (Worthington Biochemical Corp., Freehold, N. J.) or bovine α-chymotrypsin (EC 3.4.4.5) (Worthington Biochemical Corp.) were dissolved in 0.2 ml phosphate-buffered saline and added to the bacterial suspension to a final enzyme concentration of 10 μg/1.4 ml. In other experiments, 0.2 ml of granule extract from human PMN, prepared by freezing and thawing granules in 0.15 M NaCl as previously described (10), was added to give a final protein concentration of 118 μg/1.4 ml. Control tubes received 0.2 ml PBS alone. At appropriate time intervals, 0.2-ml portions of the incubation mixtures were removed and immediately mixed with 0.5 ml of 5% (wt/vol) TCA. After precipitates were allowed to develop for 30 min, all samples were centrifuged at 3,000 *g* for 10 min, and the TCA supernates were then prepared for counting. Separate zero time samples were removed into TCA immediately after mixing bacteria with enzymes and served as background corrections for each incubation mixture employed. (Background counts were essentially the same for all incubation mixtures.) Increments in TCA-soluble radioactivity over the background values were then determined and the percent of total radioactivity released as TCA-soluble counts were calculated for each time interval. Total radioactivity present in the original bacterial suspension was assayed as previously described (5). Preparation of TCA samples for counting and method of isotope counting have also been detailed in our previous publications (5).

Experiments with Viable PMN

Singly or doubly labeled *E. coli* (see above), treated with CBZ-Gly-Leu-Ala-CMK or methanol alone, were resuspended in HBSSG to give a final concentration of 2.0×10^9 bacteria/0.1 ml. Aliquots (0.1 ml) of bacterial suspension were added to siliconized glass scintillation vials containing 0.05 ml of fresh human serum. The mixtures were allowed to stand at 4°C for 10 min to permit opsonization of the bacteria, after which 0.4 ml of suspended leukocytes (prepared as described above) was mixed with each sample. The vials were incubated at 37°C in a gyrorotary water bath at 150 rpm.

Assay of protein digestion. At appropriate times, the vials were removed from the water bath and their contents transferred into centrifuge tubes. Each vial was rinsed twice with HBSSG (0.45 ml and 0.5 ml) and the rinses were also added to the centrifuge tubes. After centrifuging at 180 *g* for 10 min, the supernates were mixed with TCA to give a final TCA concentration of 5% (wt/vol) and allowed to stand for 1 h at room temperature. The mixtures were then centrifuged at 12,000 *g* for 10 min and the final TCA supernates were separated and counted for radioactivity. TCA-soluble radioactivity was expressed as percent of the total radioactivity originally present in the incubation mixture. 1.0 ml of the TCA supernates were counted after mixing with 10.0 ml Aquasol (New England Nuclear, Boston, Mass.). Total starting radioactivity was determined after overnight incubation (56°C) of 0.1 ml suspended *E. coli* in 1.0 ml Protosol (New England Nuclear). After neutralization of the mixture of bacteria and Protosol, 10 ml Aquasol was added and the resultant solution was counted. Disintegrations of ^{3}H or of ^{14}C were measured with a Mark II Liquid Scintillation Counter (Nuclear-Chicago Corp., Des Plaines, Ill.) and an Olivetti Programma 101 (Olivetti Underwood Corp., New York) (Mark II program 940016 for low ^{3}H external standard ratio and Mark II program 940018 for dual-label ^{3}H and ^{14}C external standard ratio).

Assay of PMN enzyme activities. At the end of incubation intervals, the leukocyte pellets collected at 180 *g* for 10 min were washed twice with HBSSG and resuspended in 1.0 ml of distilled water. These suspensions were individually transferred to a ground glass mortar and pestle and homogenized (125 strokes) at 4°C. Homogenates were monitored by phase microscopy for completeness of cell breakage, clarified at 17,000 *g* for 10 min, and adjusted to 0.15 M NaCl concentrations. Protein concentration of the extracts was determined by the method of Lowry et al. (11) with bovine serum albumin as a standard.

Elastase activity of the extracts was measured with *N*-acetyl-L-alanyl-L-alanyl-L-alanine-*p*-nitroanilide (AcAla$_3$-NA) (Chemalog, Plainfield, N. J.), as substrate. The assay method used was a modification of that described by Feinstein et al. (12). Sodium phosphate buffer (0.9 ml, 0.1 M, pH 7.5), 0.3–0.5 ml test sample, and 0.1 ml substrate solution (7.6 mM AcAla$_3$Na, dissolved in dimethyl sulfoxide) were mixed in a 1.5-ml cuvette. The absorbance of released *p*-nitroanilide was measured at 410 nM with a Gilford recording spectrophotometer (Model 2400-S, Gilford Instrument Laboratories, Inc., Oberlin, Ohio). Controls containing saline in place of cell extract were always included to correct for spontaneous substrate breakdown. Enzyme activity was expressed as the change in absorbance/15 min/250 μg of extract protein.

β-Glucuronidase activity was determined by a modification of the method of Brittinger et al. (13). The assay mixture included 1.0 ml acetate buffer (0.1 M, pH 4.6), 0.3 ml test sample, and 0.1 ml substrate (10 mM phenolphthalein glucuronic acid, Sigma Chemical Co., St. Louis, Mo.). The incubation was carried out in a water bath at 37°C for 18 h. Enzyme activity was expressed as micrograms phenolphthalein released (calculated from a calibration plot) per 100 μg extract protein at the end of the incubation period.

RESULTS

Pilot experiments with soluble enzymes. Before incubating *E. coli* pretreated with chloromethyl ketone enzyme-inactivators with living neutrophils, several pilot experiments were done to determine if bacteria treated in this way could inhibit protein digestion by soluble enzymes in a cell-free test system. To accomplish this, *E. coli* pretreated with chloromethyl ketone inactivators or methanol alone were incubated with two crystalline enzymes, bovine α-chymotrypsin or porcine pancreatic elastase, or with crude extracts of human leukocyte granules. Digestion of *E. coli* proteins by the crystalline enzymes or the granule extract was measured as the percent of total [^{3}H]arginine initially present in the bacterial cells, which became released into the TCA-soluble phase of the incubation medium (see Methods).

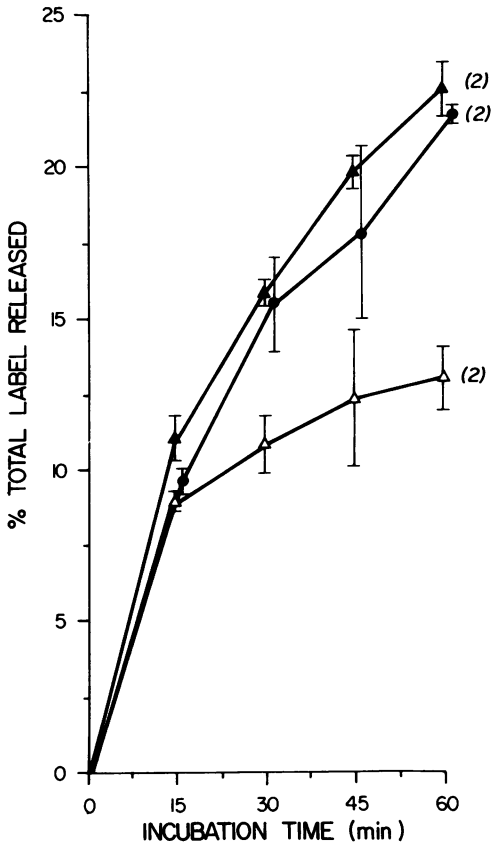

FIGURE 1 In vitro digestion of [^{3}H]arginine-labeled *E. coli* by α-chymotrypsin. 10 μg enzyme was used per incubation mixture (1.4 ml). The incubation buffer was 0.05 M Tris-HCl, pH 7.6, at 37°C, containing 0.1 M NaCl and 1 mM $CaCl_2$. ▲——▲, *E. coli* pretreated with methanol; ●——●, *E. coli* pretreated with CBZ-Gly-Leu-Ala-CMK (elastase inactivator) dissolved in methanol; △——△, *E. coli* pretreated with CBZ-Gly-Leu-Phe-CMK (chymotrypsin inactivator) dissolved in methanol. Numbers in parenthesis are the number of experiments. Vertical lines represent the SD. Background release (no enzyme) was less than 1.0% after 60 min with all three bacterial preparations.

Fig. 1 shows the results of two experiments in which chymotrypsin was incubated with *E. coli* previously treated with CBZ-Gly-Leu-Phe-CMK (a chymotrypsin inactivator, see ref. 6), CBZ-Gly-Leu-Ala-CMK (an elastase inactivator, see ref. 6), or methanol alone. As shown in the figure, there was inhibition of digestion by the enzyme at 30, 45, and 60 min of incubation, when CBZ-Gly-Leu-Phe-CMK-treated bacteria were used. In contrast, when chymotrypsin was incubated with *E. coli* treated with CBZ-Gly-Leu-Ala-CMK, there was no change in the release of TCA-soluble label from that observed in the case of methanol-treated bacteria. The absence of inhibition by the chymotrypsin inactivator at the early time period may be because chloromethyl ketones, although irreversable inactivators, are nonetheless slow-acting (14).

Fig. 2 represents the results of two experiments using pancreatic elastase and the same three preparations of *E. coli* described above. In these experiments, digestion of *E. coli* proteins at later time periods (45 and 60 min) appears to be partly inhibited in the organisms pretreated with CBZ-Gly-Leu-Ala-CMK, the elastase inactivator.

Fig. 3 shows the results obtained when crude extract of human leukocyte granules was incubated with *E. coli* treated with CBZ-Gly-Leu-Phe-CMK, CBZ-Gly-Leu-Ala-CMK, or methanol. A modest inhibition of digestion occurred when bacteria pretreated with elastase inactivator were used as substrate for the leukocyte granules, reminiscent of that seen when pancreatic elastase was the digestive enzyme (compare with Fig. 2). There was no inhibition when bacteria pretreated with chymotrypsin inactivator were used. (However, it should be noted that the granule extract used in this experiment was prepared with 0.15 M NaCl, as opposed to higher ionic strength buffer, and that under these conditions relatively little chymotrypsin is extracted from granule membranes [9]. Therefore, the failure of CBZ-Gly-Leu-Phe-CMK to inhibit bacterial digestion by such extracts [Fig. 3] should not be taken as evidence against the participation of granulocyte chymotrypsin in bacterial degradation within viable leukocytes.) In any case, the results shown in Figs. 1–3

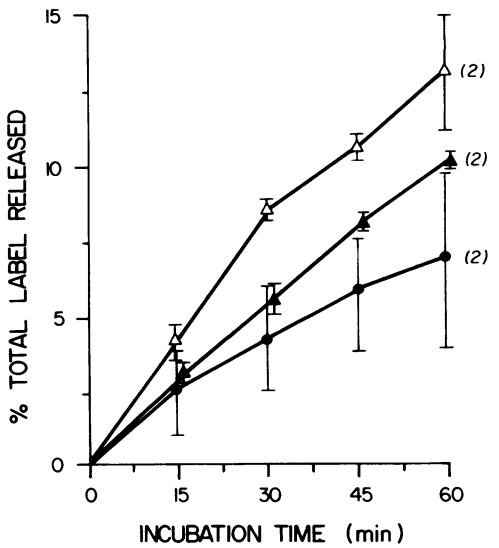

FIGURE 2 In vitro digestion of [^{3}H]arginine-labeled *E. coli* by elastase. Enzyme concentrations and buffer are as in Fig. 1. ▲——▲, *E. coli* pretreated with methanol; ●——●, *E. coli* pretreated with CBZ-Gly-Leu-Ala-CMK (elastase inactivator) dissolved in methanol; △——△, *E. coli* pretreated with CBZ-Gly-Leu-Phe-CMK (chymotrypsin inactivator) dissolved in methanol. Numbers in parenthesis are the number of experiments. Vertical lines represent the SD. Background release (no enzyme) is less than 1.0% after 60 min with all three bacterial preparations.

suggested that the chloromethyl ketone inactivators remaining associated with the treated *E. coli* were at least partly active against their specific proteolytic enzymes.

Experiments with living leukocytes treated with a metabolic poison. Before incubating inactivator-pretreated *E. coli* with living leukocytes, an additional pilot experiment was performed. This experiment was designed to test the sensitivity of our method for measuring the release of isotope from viable leukocytes after ingestion of labeled bacteria and the validity of this method as a test of leukocyte phagocytic or digestive functions. Leukocytes were preincubated with sodium arsenite (0.2 mM) for 10 min at 37°C. Leukocytes preincubated at 37°C for 10 min without sodium arsenite served as a control. Labeled methanol-treated *E. coli* were added to both leukocyte suspensions and the release of TCA-soluble label was measured (see Methods). Sodium arsenite is a partial inhibitor of phagocytosis by PMN (15); therefore, one would expect to find partial inhibition of the release of TCA-soluble label in the leukocytes pretreated with this agent. The concentration of arsenite used has been reported (15) to reduce oxygen consumption to approximately 50% of control values and glucose consumption to 12% of control values in rabbit peritoneal PMN. These reductions in oxygen and glucose consumption were associated with a 30% inhibition of phagocytosis by arsenite-treated leukocytes (15). As shown in Fig. 4, the results were as predicted, suggesting that our assay system might prove sufficiently sensitive to detect inhibition of *E. coli* protein digestion, when inactivator-pretreated organisms were fed to viable phagocytes.

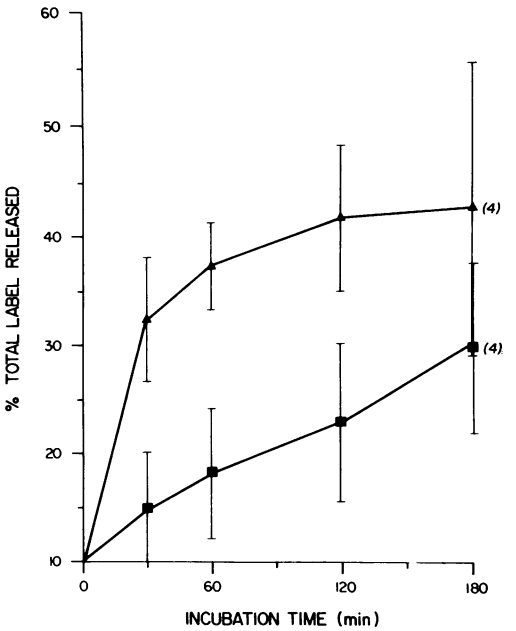

FIGURE 4 Digestion of [^{3}H]arginine-labeled *E. coli* by living leukocytes with and without sodium arsenite. ▲——▲, no sodium arsenite; ■——■, sodium arsenite (0.2 mM). Numbers in parenthesis are the number of experiments. Vertical lines represent SD. ($P < 0.05$, Student's *t* test).

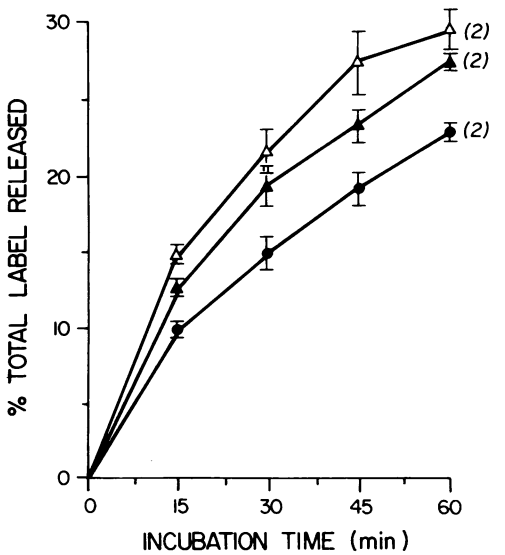

FIGURE 3 In vitro digestion of [^{3}H]arginine-labeled *E. coli* by leukocyte granule extract. 118 μg granule protein was used per incubation mixture (1.4 ml). Other conditions are as in Fig. 1. ▲——▲, *E. coli* pretreated with methanol; ●——●, *E. coli* pretreated with CBZ-Gly-Leu-Ala-CMK (elastase inactivator) dissolved in methanol; △——△, *E. coli* pretreated with CBZ-Gly-Leu-Phe-CMK (chymotrypsin inactivator) dissolved in methanol. Numbers in parenthesis are the number of experiments. Vertical lines represent the SD. Background release (no enzyme) is less than 1.0% after 60 min with all three bacterial preparations.

Experiments with living leukocytes and inactivator-pretreated bacteria. [^{3}H]Arginine-labeled *E. coli*, pretreated either with CBZ-Gly-Leu-Ala-CMK dissolved in methanol or with methanol alone, were incubated with living human neutrophils. Fig. 5 represents the TCA-soluble isotope release, expressed as percent of total bacterial label present. At 60 and 120 min of incubation, significant inhibition of isotope release occurred in the leukocytes incubated with *E. coli* treated with CBZ-Gly-Leu-Ala-CMK. The inhibition at both time intervals was 24%. The absence of inhibition at 30 min may be explained by the slow action of chloromethyl ketone inactivators (14). The absence of inhibition at 120 min may be due to the activity of other, later-acting proteases in the leukocyte digestive vacuole, which were unaffected by the elastase inactivator. *E. coli* (control and pretreated) released less than 1% of the label when incubated in the absence of leukocytes.

We next carried out an experiment to test the possibility that the result shown in Fig. 5 was due to the

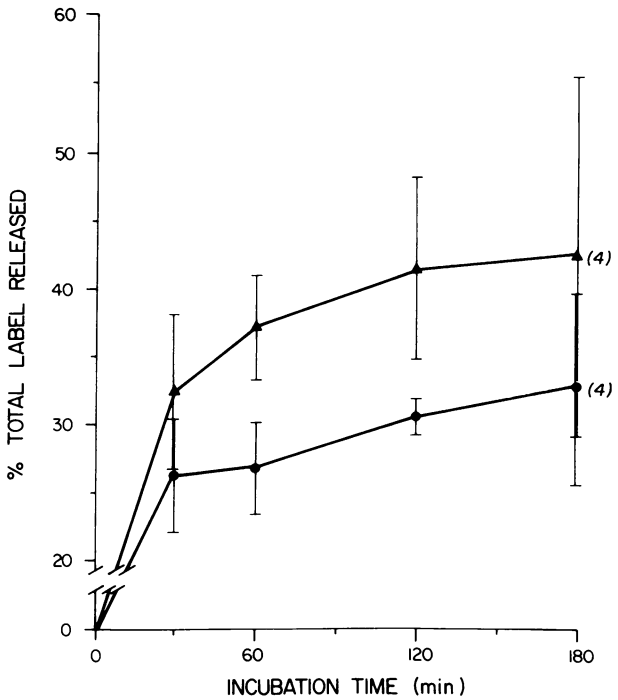

FIGURE 5 Digestion of [^{3}H]arginine-labeled *E. coli* by viable leukocytes. ▲——▲, *E. coli* pretreated with methanol; ●——●, *E. coli* pretreated with CBZ-Gly-Leu-Ala-CMK (elastase inactivator) dissolved in methanol. Numbers in parenthesis are the number of experiments. Vertical lines represent SD. At incubation times of 60 and 120 min, $P < 0.05$ (Student's t test).

effect of CBZ-Gly-Leu-Ala-CMK upon phagocytosis of treated microorganisms, rather than to its effect upon protein digestion within PMN. *E. coli* were doubly labeled with [^{14}C]arginine and [^{3}H]thymidine and treated with CBZ-Gly-Leu-Ala-CMK or with methanol as before. When such cells were fed to viable PMN, release of TCA-soluble ^{3}H was the same whether inactivator was present or not (Fig. 6). The activity of leukocyte DNAase should not be affected by CBZ-Gly-Leu-Ala-CMK. Thus, inhibition of ^{3}H-release could only have resulted if phagocytosis or degranulation were blocked by the inactivator. That this did not occur suggests that phagocytosis and degranulation were not affected. On the other hand, release of TCA-soluble ^{14}C was inhibited (Fig. 7), suggesting that the specific elastase inactivator had selectively interfered with digestion of bacterial protein. It is of interest to note that percent inhibition of ^{14}C release at 30, 60, and 90 min of incubation was 24%, identical to the percent inhibition of isotope release observed in the preceding experiment (Fig. 5), where ^{3}H had been used to label the bacterial protein.

A final experiment was then carried out to measure the effect of inactivator-pretreated *E. coli* upon lysosomal enzyme activities within leukocytes that had phagocytized these microorganisms. PMN were incubated with methanol-treated bacteria or with chloromethyl ketone-treated bacteria as before, and the leukocytes were then homogenized as described under Methods.

Activities of β-glucuronidase and elastase were assayed in the post-granule supernatant fraction of the homogenates. This fraction contains enzymes released into digestive vacuoles, which become ruptured during homogenization of the cells. The results of the enzyme assays are given in Table I. It can be seen that activity of β-glucuronidase was essentially unaffected by inactivator-treated bacteria, whereas activity of the post-granule supernate upon an elastase-specific amide substrate was depressed by the inactivator-treated microorganisms. (As is evident from the table, enzyme levels varied from one leukocyte preparation to another, but the trend of the results [absence of inhibition vs. inhibition] was constant.) The results of the β-glucuronidase assays suggest that the discharge of lysosomal hydrolases into the digestive vacuoles of phagocytising leukocytes was the same, whether the leukocytes had engulfed control or inactivator-pretreated *E. coli*. Since elastase and β-glucuronidase have been localized to the same class of lysosomes in human PMN (7), the lower elastase activity found in homogenates of PMN feeding on in-

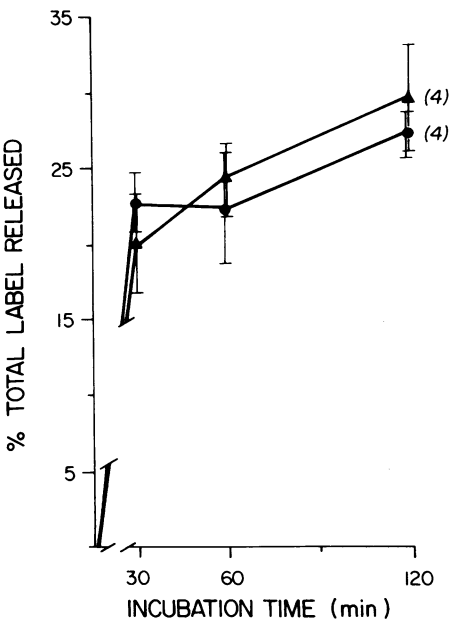

FIGURE 6 Digestion of *E. coli*, doubly labeled with [^{3}H]-thymidine and [^{14}C]arginine, by viable leukocytes. Release of TCA-soluble ^{3}H is shown. ▲——▲, *E. coli* pretreated with methanol; ●——●, *E. coli* pretreated with CBZ-Gly-Leu-Ala-CMK (elastase inactivator) dissolved in methanol. Numbers in parenthesis are the number of experiments. Vertical lines represent SD.

activator-treated *E. coli* probably reflects selective inhibition of this enzyme.

DISCUSSION

Our purpose in carrying out these experiments was to evaluate the participation of elastases in the digestion of bacterial proteins by living human PMN leukocytes. Earlier studies from our laboratory (5) had suggested that PMN elastases contribute significantly to *E. coli* protein digestion, but those studies were limited by the constraints of an artificial test system employing sonicated bacteria and PMN granule extracts. Our present system allows the use of viable PMN and intact *E. coli*, and therefore more closely approximates the situation found in vivo. With this system, our results show that a significant fraction of *E. coli* protein is digested by PMN leukocyte elastase, and thus support the conclusions of our previous studies (5).

The inhibition of digestion obtained when *E. coli* are pretreated with elastase inactivator and then incubated with viable PMN is less than the inhibition obtained in our earlier studies (5). This observation may be explained by the fact that chloromethyl ketones, although specific and irreversible, are slow-acting (14). In our earlier experiment (5), granule extracts were preincubated for up to 2 h with inactivator and only then mixed with the labeled bacterial protein substrate. In contrast, in the present experiments, the substrate, rather than the enzyme, was pretreated with inactivator and both substrate and inactivator were then simultaneously presented to the leukocyte digestive apparatus. This important difference in methodology could account for the different degrees of inhibition observed in our earlier and present studies. In addition, other leukocyte proteases may also participate in *E. coli* protein digestion besides elastase, and their action could also partly explain the incomplete inhibition achieved with elastase inactivators. Alternative proteases could include PMN collagenases (16), which show affinity for other proteins besides collagen (17), and chymotrypsin-like proteases, which also have broad substrate affinities (9, 18–20). Experiments on the role of PMN-chymotrypsin in *E. coli* digestion by viable leukocytes will be reported in a separate publication.[3]

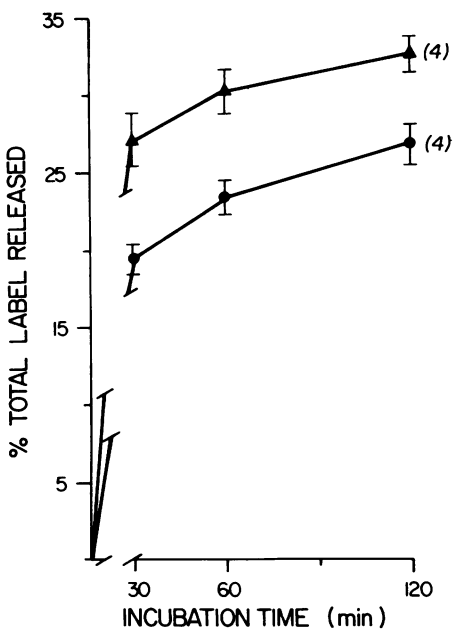

FIGURE 7 Digestion of *E. coli*, doubly labeled with [^{3}H]-thymidine and [^{14}C]arginine, by viable leukocytes. Release of TCA-soluble ^{14}C is shown. (Same experiment as in Fig. 6.) ▲——▲, *E. coli*, pretreated with methanol; ●——●, *E. coli* pretreated with CBZ-Gly-Leu-Ala-CMK (elastase inactivator) dissolved in methanol. Numbers in parenthesis are the number of experiments. Vertical lines represent SD, $P < 0.01$ (Student's t test).

TABLE I

Enzyme Activities in Post Granule Supernatant Fractions of Homogenized Leukocytes after 30 min Incubation with E. Coli

Experiment number	Pretreatment of *E. coli*	β-Glucuronidase*		Elastase‡	
		μg/18 h/100 μg protein			
1	Methanol	13.7		0.049	
	CBZ-Gly-Leu-Ala-CMK dissolved in methanol	11.9	(−13.0%)	0.029	(−41%)
2	Methanol	27.5		0.051	
	CBZ-Gly-Leu-Ala-CMK dissolved in methanol	31.9	(+16.0%)	0.024	(−53%)
3	Methanol	19.6		NT	
	CBZ-Gly-Leu-Ala-CMK dissolved in methanol	19.0	(−3.0%)	NT	
4	Methanol	22.6		0.223§	
	CBZ-Gly-Leu-Ala-CMK dissolved in methanol	26.0	(+15.0%)	0.045§	(−80.0%)
Mean			(+4.0%)		(−58.0%)

Number in parenthesis is percent inhibition; NT = not tested.

* β-glucuronidase activity units: μg phenolphthalein released per 18 h per 100 μg supernatant protein (substrate was phenolphthalein glucuronic acid).

‡ Elastase activity units: change in absorbance at 410 nm per 15 min per 250 μg supernatant protein (substrate was $AcAla_3NA$).

§ In exp. 4, a different $AcAla_3NA$ substrate preparation was used than in exps. 1 and 2. This new substrate was six times more sensitive to a standard preparation of porcine pancreatic elastase than the substrate used earlier.

[3] Blondin, J., and Janoff, A. Manuscript in preparation.

The elastase-inactivator employed (CBZ-Gly-Leu-Ala-CMK) is specific for elastases, as shown by the work of independent investigators (6). In addition, we observed the inability of this agent to inactivate bovine α-chymotrypsin (see Fig. 1). Therefore, it seems likely that the inhibition observed when this agent was used reflects the participation of PMN elastases in intracellular digestive processes of human leukocytes. Nevertheless, the specificity of chloromethyl ketones for their respective enzymes notwithstanding, there is also the possibility that the observed inhibition of *E. coli* protein digestion by the elastase inactivator was due to nonspecific suppression of cellular chemotaxis, phagocytosis, or degranulation mechanisms. Our experiments with doubly labeled *E. coli* ([^{3}H]thymidine and [^{14}C]arginine) appear, however, to rule out all three possibilities. These data show that the release of TCA-soluble ^{3}H is unaffected by pretreatment of bacteria with the elastase inactivator (Fig. 6), whereas the release of TCA-soluble ^{14}C is simultaneously inhibited, thus suggesting that bacterial protein digestion is suppressed, but that degradation of bacterial DNA proceeds at a normal rate. The latter conclusion, in turn, favors the interpretation that PMN chemotaxis, phagocytosis, and degranulation are unaffected by the chloromethyl ketone.

Further evidence against inhibition of degranulation by the inactivator was obtained from the enzyme assays summarized in Table I. These measurements were carried out on the post-granule supernatant fraction of homogenized leukocytes, after the cells had phagocytized either *E. coli* treated with CBZ-Gly-Leu-Ala-CMK or methanol. Two enzymes (β-glucuronidase and elastase), localized in the same class of PMN granules in man (7), were assayed. Post-granule supernatant fractions should contain all of the nonsedimentable, lysosomal enzyme activity solubilized through degranulation. As expected, elastase activity in the post-granule supernatant fraction was depressed in the PMN that had phagocytized *E. coli* treated with elastase inactivator, in comparison to leukocytes that had phagocytized *E. coli* treated with methanol (Table I). In contrast, β-glucuronidase activity was the same, whether the PMN had phagocytized inactivator-pretreated or methanol-pretreated *E. coli* (Table I). The finding of control levels of β-glucuronidase activity in the post-granule supernatants derived from leukocytes exposed to the inactivator argues that degranulation mechanisms were not suppressed by the chloromethyl ketone.

In these experiments, no effort was made to prove conclusively that the elastase inactivator was physically associated with the treated bacterial cells, although in preliminary trials (see Methods), repeated washings of treated *E. coli* with aqueous buffers failed to remove inactivator but dissociation did occur when methanol was used as a wash medium. In any event, it is not essential to our thesis to know whether the inactivator was attached to the microorganisms or merely co-suspended with them, since simultaneous endocytosis of inactivator and bacteria (whether associated or not) satisfies the experimental design.

Other laboratories have also studied bacterial digestion by living PMN. In general, these studies (21–23) have examined digestion products produced by the entire complement of granule enzymes, but have not attempted to elucidate the specific enzyme(s) responsible for the leukocyte's digestive processes. A notable exception to this was the study by Elsbach et al. (24) on the role of rabbit PMN phospholipase A in bacterial digestion by living leukocytes. Our system, involving association of specific enzyme-inactivators with bacteria containing labeled target macromolecules, allows examination of the role of individual enzymes in the leukocyte's digestive process. This experimental model may find application in studies of the degradation of other bacterial components, besides proteins.

The results given in this paper suggest that PMN elastase participates in *E. coli* digestion, once the microbes have been ingested and killed by the leukocytes. Our data do not shed any light on the possibility of a direct bactericidal role for this leukocyte enzyme. Indeed, the viability of our test organisms can be questioned in view of their pretreatment with methanol and storage at − 20°C, so that measurement of intracellular killing cannot be evaluated in our experimental system. Moreover, it has recently been reported (25) that the bactericidal properties of human PMN chymotrypsin-like enzymes are independent of their enzymatic function. For example, thermal inactivation of PMN chymotrypsins does not affect the microbicidal potency of these leukocyte proteins. Should PMN elastase (like the chymotrypsins) normally exert any bactericidal activity, forming a complex of the enzyme with an active site-specific chloromethyl ketone might not necessarily destroy its bactericidal properties. We therefore limit our conclusions to the digestive role of PMN elastase and offer no interpretation, based on these studies, of its possible microbicidal function in neutrophil physiology.

In summary, the data presented in this paper suggest that one function of elastases within human PMN is their role in the digestion of *E. coli* proteins. Further studies with other species of bacteria are now required to extend this hypothesis of leukocyte elastase physiology.

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