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The Role of Elastase in the Digestion of *E. coli* Proteins by Human Polymorphonuclear Leukocytes

I. Experiments *in Vitro*[1] (38027)

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Our laboratory was the first to demonstrate elastase-like esteroprotease activity in granule fractions prepared from human polymorphonuclear leukocytes (PMN) (1, 2). Recent work by others suggests that such enzymes constitute the major esterases of human PMN (3). To date, three elastases have been found in these cells by acrylamide gel electrophoresis techniques (3) and by affinity chromatography (4). Such enzymes could participate in elastin damage during pathological processes associated with acute inflammation (5). However, the physiological functions of the PMN elastases remain unknown. We recently suggested (6) that PMN elastases might provide a bacterial wall lysing function to complement that of PMN lysozyme. Such a function was proposed for bacterial species with peptidoglycan cross-linkages containing amino acid sequences related to those present in tropoelastin or in the cross-links of elastin fibers.

On the other hand, since elastases have broad proteolytic activity in addition to their elastolytic action, the PMN elastases could also participate in the digestion of a variety of bacterial proteins regardless of the initial mechanism of PMN attack upon bacterial cell walls. Therefore, the present experiments were undertaken to measure the participation of elastases in the digestion of *E. coli* proteins by human PMN granule extracts *in vitro*. Digestion was, in the main, carried out at neutral pH, in view of the evidence that the pH of human PMN digestive vacuoles remains near neutrality (7). We employed active-site directed, irreversible chloromethyl ketone elastase inhibitors (8) to inactivate the enzymes in question and compared granules treated in this way with untreated granules. Using this approach, the complete role of the elastases in *E. coli* protein digestion could be evaluated; that is, elastase-mediated digestion occurring independently of other granule enzymes, as well as digestion requiring the cooperative action of other enzymes of the leukocyte granule but still dependent on elastase-mediated cleavages.

Materials and Methods. E. coli, strain K-MX74T2, was obtained from Dr. M. Inouye (Stony Brook University) and grown in M9 medium supplemented with 4 μg/ml thymidine and 2 μg/ml thiamine. When the organisms entered logarithmic growth phase, 10 μCi of ^{3}H-arginine were added to the culture in the presence of an excess of unlabeled arginine (10 μg/ml). Growth was maintained in the presence of labelled arginine for two additional generation times (about 80 min) and was then terminated by rapidly chilling the cells to 0°, removing them from the medium by centrifugation (12,000*g* × 10 min) and transferring them to buffer as indicated below. In order to preserve uniformity in all experiments, growth times were adjusted so that the culture's Klett reading (1 cm light path, blue filter) was always = 0.15 at the time the cells were harvested. A constant volume of culture medium (35 ml) was then used to prepare the sonicated *E. coli* suspension for each experiment. After sedimentation out

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of the culture medium, the bacteria were washed three times in 25 ml of cold, phosphate-buffered saline (pH 7) containing 0.1% unlabelled arginine and were left in 25 ml of the buffer–arginine solution overnight at 4°. In this way, ^{3}H-arginine present in the bacterial amino acid pool but not incorporated into protein could be largely eliminated from the cells by exchange with arginine in the medium. After a final centrifugation, the washed cells were suspended in 8 ml of 0.05 *M* Tris–HCl buffer (pH 7.6 at 37°) containing 0.1 *M* NaCl and 0.001 *M* $CaCl_2$. The suspension was placed in ice-water and subjected to three, spaced, 1 min periods of sonication at 50 W energy output (Sonifier Cell Disrupter, Model W140, Ultrasonics Inc., Plainview, NJ). Nearly complete cell breakage was achieved as monitored by phase-contrast microscopy.

Human PMN granule extracts were obtained essentially as described before (1). *N*-Acetyl-L-alanyl-L-alanyl-L-alanine chloromethyl ketone (AAACK) and *N*-acetyl-L-alanyl-L-alanyl-L-prolyl-L-alanine chloromethyl ketone (AAPACK) were synthesized and provided by Drs. J. Powers and P. M. Tuhy of the Georgia Institute of Technology. These agents have been reported as being highly specific elastase inhibitors (8). *N*-α-Tosyl-L-lysine chloromethyl ketone (TLCK), which inhibits trypsin but not elastase (at the concentrations used in these experiments) was purchased from Cyclo Chemical Corp., Los Angeles, California and was used as a control. Preincubation of PMN granule extracts with 0.002 *M* inhibitors was carried out in pH 7 phosphate buffered saline for 2 hr at room temperature.

Mixtures of sonicated bacteria and granule extract were incubated together at 37°, and 0.2 ml aliquots removed at different times into tubes containing 0.5 ml of 5% (w:v) TCA. Bacteria incubated alone served as a control for autodigestion. Untreated granule extracts were compared with granule extracts pretreated with one of the three chloromethyl ketone inhibitors. Sample times and concentrations of granule protein are given in the Results. Separate zero-time samples, which were removed into TCA immediately after mixing chilled sonicate with granule extract, served as background corrections for each incubation mixture employed. (These corrections were essentially the same in all incubation mixtures.) Increments in TCA-soluble radioactivity over this background value were then determined and the percent of total radioactivity released was calculated for each time interval. Measurement of total radioactivity in the original sonicate was performed on samples hydrolyzed for 16 hr in 8 *N* HCl at 110°. TCA supernatants were prepared for counting by centrifuging all samples at 3000*g* × 10 min after allowing precipitates to form for 30 min at room temperature. Acidity of each sample was then neutralized with NaOH in the presence of phenolphthalein indicator before scintillation counting was carried out. DPM (^{3}H) were measured in Bray's solution using a Mark II Liquid Scintillation Counter (Nuclear Chicago) and an Olivetti Programma 101 (Mark II program 940016 for low ^{3}H ESR).

Percent inhibition of protein digestion was calculated from the values obtained at 10 min of incubation, since the rate of digestion given by untreated granule extract remained relatively constant during this interval (Fig. 1).

Results. Figure 1 shows the results of the experiments described above. The percent inhibitions calculated from the data in Fig. 1 are summarized in Table I. It is clear from these data that a major portion of the proteolytic activity of human PMN granules, at neutral pH, upon ^{3}H-arginine labelled *E. coli* proteins *in vitro* is dependent upon the action of PMN elastases. A chloromethyl ketone inhibitor without specificity for elastases (TLCK) had no significant inhibitory effect upon proteolysis by PMN granules. By contrast, both of the specific chloromethyl ketone elastase inhibitors (AAACK and AAPACK) produced significant inhibition (Table I).

In one experiment, simultaneous incubations were carried out in 0.05 *M* citrate buffer at pH 3 (data not shown). Under these conditions, there was no significant

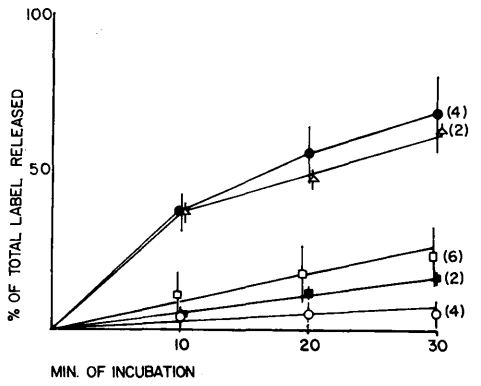

FIG. 1. ●, Sonicate + human PMN granule extract (S + G); □, S + G pretreated with 0.002 *M* AAACK; ■, S + G pretreated with 0.002 *M* AAPACK; △, S + G pretreated with 0.002 *M* TLCK; ○, Bacterial sonicate alone. Incubation conditions: 34 μg/ml PMN granule protein, pH 7.6, 37°, final reaction volume = 1.4 ml. No. in parentheses = total experiments. Vertical lines represent SD.

release of labelled peptides by the granule protein. Thus, after 3 hr of incubation at acid pH, the release from bacterial sonicate incubated alone was 5% of total incorporated label, while release in the presence of granule extract was only 10%. In contrast to this, when the reaction was carried out in pH 7.6 buffer, 10% release of labelled peptides had already occurred after 3 min (see Fig. 1). This finding correlates with previous observations by ourselves and others on the pH-activity profile of human PMN granule proteases. Proteolytic activity of human PMN extracts at neutral or slightly alkaline pH greatly exceeds that occurring at acid pH (9).

Discussion. Our purpose in carrying out these experiments was to evaluate the participation of elastase-like enzymes in the *in vitro* digestion of *E. coli* proteins by human PMN granule extracts. Our results show that a major portion of this digestive activity (at neutral pH) is derived from the elastase-like esteroproteases. No significant digestion of *E. coli* protein occurs at pH 3.0.

The absence of any significant inhibition of *E. coli* protein digestion by TLCK supports the conclusion that the inhibitions obtained with AAACK and AAPACK reflect specific suppression of PMN elastases.

TABLE I. Chloromethyl Ketone Inhibition of *E. coli* Protein Digestion by Human PMN Granules.[a]

Inhibitor	Conc. (*M*)	Inhibition at 10 min[b,c] (%)
TLCK	.002	0
AAACK	.002	82
AAPACK	.002	94

[a] Data derived from Fig. 1.

[b] 10 min corresponds to linear phase of digestion reaction.

[c] 100% inhibition corresponds to rate of labeled peptide release observed with bacterial sonicate alone.

These studies with specific inhibitors of the enzymes in question permit evaluation of the *total* elastase contribution to proteolysis, including elastase-dependent proteolytic steps which require prior or subsequent cleavages by other granule enzymes in order for TCA-soluble peptides to be released.

Inspection of Fig. 1 shows that, despite the marked inhibition of protein digestion in the presence of elastase inhibitors, some degradation does proceed at a steady (albeit reduced) rate leading to a modest release of labelled peptides by 30 min. This level of release is significant in comparison with the control bacteria. Thus, it is likely that other neutral proteases of the human PMN granule also participated in breakdown of *E. coli* proteins under our *in vitro* conditions. Among the latter enzymes could be the neutral collagenases previously described in human PMN granules (10), especially since these enzymes also show affinity for some other proteins besides collagen (11). 0.01 *M* EDTA, which inhibits PMN collagenase (10) but not PMN elastase (12), was added to reaction mixtures in two of our experiments, resulting in a small inhibition of labelled peptide release. However, these results are preliminary and further study is required to elucidate the role of collagenases or other divalent cation-dependent enzymes in digestion of *E. coli* proteins.

In conclusion, our data indicate that an important physiologic role may be served

by the elastase-like enzymes of human PMN; namely, the digestion of proteins of phagocytosed bacteria. Further studies with other bacteria besides *E. coli,* and experiments on the effect of elastase-inhibitors upon digestion of bacteria within living PMN will be required to gain fuller insight into this important question.

Summary. E. coli proteins were labelled by culturing the organisms in the presence of ^{3}H-arginine. Sonicated preparations of bacteria were then incubated with water-soluble extracts of human PMN granules, and the rate of release of labelled peptides into the TCA-soluble phase was determined under different experimental conditions. Release was negligible at pH 3, but occurred at a significant rate at pH 7.6. Release at this pH was inhibited 82% and 94% by preincubation of the granule extract with 0.002 *M* AAACK or AAPACK respectively. The latter agents are specific, active-site directed, irreversible chloromethyl ketone elastase inhibitors. Granule extract preincubated with 0.002 *M* TLCK, a chloromethyl ketone trypsin inhibitor, retained full digestive activity against *E. coli* proteins. These *in vitro* results suggest that the elastase-like enzymes may contribute significantly to *E. coli* protein digestion following phagocytosis of this organism, and perhaps of other bacterial species, by human PMN.

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