

## Antibacterial Activity of Antileukoprotease

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**Antileukoprotease (ALP), or secretory leukocyte proteinase inhibitor, is an endogenous inhibitor of serine proteinases that is present in various external secretions. ALP, one of the major inhibitors of serine proteinases present in the human lung, is a potent reversible inhibitor of elastase and, to a lesser extent, of cathepsin G. In equine neutrophils, an antimicrobial polypeptide that has some of the characteristics of ALP has been identified (M. A. Couto, S. S. L. Harwig, J. S. Cullor, J. P. Hughes, and R. I. Lehrer, *Infect. Immun.* 60:5042-5047, 1992). This report, together with the cationic nature of ALP, led us to investigate the antimicrobial activity of ALP. ALP was shown to display marked in vitro antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*. On a molar basis, the activity of ALP was lower than that of two other cationic antimicrobial polypeptides, lysozyme and defensin. ALP comprises two homologous domains: its proteinase-inhibitory activities are known to be located in the second COOH-terminal domain, and the function of its first NH<sub>2</sub>-terminal domain is largely unknown. Incubation of intact ALP or its isolated first domain with *E. coli* or *S. aureus* resulted in killing of these bacteria, whereas its second domain displayed very little antibacterial activity. Together these data suggest a putative antimicrobial role for the first domain of ALP and indicate that its antimicrobial activity may equip ALP to contribute to host defense against infection.**

Proteinase inhibitors are thought to play an important role in the regulation of the extracellular action of proteinases, such as the serine proteinase human leukocyte elastase, that are released from stimulated neutrophils. Elastase can cause extensive tissue degradation and has been shown to be involved in several diseases, including pulmonary emphysema. Proteinase inhibitors involved in protecting the lung against the action of elastase include  $\alpha$ 1-proteinase inhibitor, which is produced mainly in the liver and reaches the lung by passive diffusion (35), and two locally produced inhibitors, elafin (also known as skin-derived antileukoprotease) (13, 31, 38) and antileukoprotease (ALP) (14). ALP, also known as secretory leukocyte proteinase inhibitor, is an effective inhibitor of human leukocyte elastase and to a lesser extent of cathepsin G. It is a low-molecular-weight ( $M_r$ , 11,700) cationic nonglycosylated molecule that is composed of two highly homologous domains (33); the second COOH-terminal domain contains its proteinase inhibitory site, whereas the function of the first NH<sub>2</sub>-terminal domain is largely unknown (7, 16, 26, 37). A recent report demonstrated that the NH<sub>2</sub>-terminal domain may aid in stabilizing the elastase-ALP complex and may mediate the enhancement of the antiproteinase activity of ALP by heparin (39). ALP is produced locally in bronchi by serous cells in the submucosal glands and by Clara and goblet cells of the bronchiolar and bronchial lining epithelium (17). Because it is present in different parts of the airways, ALP is thought to play an important role in maintaining the proteinase-antiproteinase balance in the central and possibly also the lower airways (11).

Both domains of ALP contain a characteristic pattern of cysteine residues; this pattern, known as the four-disulfide core (FDC), is also found in a variety of other molecules with

miscellaneous functions (6, 33). Collectively this group of proteins is often referred to as the FDC family of proteins. Recently a novel antimicrobial polypeptide from equine neutrophils, designated eNAP-2, was identified and shown to be structurally homologous to members of the FDC protein family (3). In addition, eNAP-2 displayed antiproteinase activity against microbial serine proteinases but not against mammalian proteinases (4). Given this homology of ALP with eNAP-2 and the cationic nature of ALP, we investigated the antibacterial activity of ALP.

### MATERIALS AND METHODS

**Proteins.** Recombinant ALP was produced in *Escherichia coli* and purified as previously described (29). The separated NH<sub>2</sub>- and COOH-terminal domains were obtained by partial acidic hydrolysis. Briefly, desalted ALP (ca. 15 to 20 mg/ml) was incubated in 50% (vol/vol) acetic acid in a water bath at 90°C for 1 h. The mixture was cooled on ice and then slowly titrated to pH 4.5 with 10 N NaOH. This solution was then passed over a column of octyl-Sepharose (Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated in 3 M NaCl. The NH<sub>2</sub>-terminal domain (domain 1) did not bind to the column and was recovered from the fall-through. The COOH-terminal domain (domain 2) bound to the matrix and was eluted with 50 mM sodium acetate (pH 4.5). Both fractions were desalted by diafiltration using an ultrafiltration cell filled with a membrane with a nominal cutoff of 5 kDa. They were further purified by cation-exchange chromatography on carboxymethyl-Sepharose (Pharmacia LKB Biotechnology), using 100 mM sodium acetate (pH 4.5) as the starting buffer, and eluted with a linear gradient of NaCl in the same buffer.

Defensins were purified from an acetic acid extract of purulent sputum. Briefly, 40 ml of purulent sputum was sonicated, 30% (vol/vol) acetic acid was added to a final concentration of 20%, and the material was extracted by overnight incubation at 4°C. Insoluble material was removed by centrifugation for 20 min at 27,000 × g; the cleared supernatant was dialyzed overnight against 5% acetic acid in Spectrapor 3 tubing (Spectrum Medical Instruments Inc., Los Angeles, Calif.) and concentrated to 10 ml by vacuum centrifugation (Speed-Vac; Savant Instruments Inc., Hicksville, N.Y.). The concentrated acetic acid sputum extract was fractionated by gel filtration on a Sephacryl S-200 column (2.5 by 90 cm; Pharmacia) that had been equilibrated in 5% acetic acid. The fractions were analyzed for protein content by the bicinchoninic acid method (Pierce, Rockford, Ill.) and for lysozyme activity (32) and antibacterial activity against *E. coli* ML-35p (kindly provided by R. I. Lehrer, UCLA School of Medicine, Los Angeles, Calif.) by using a radial diffusion assay (20). The low-molecular-weight fractions that eluted after lysozyme and displayed antibacterial activity against *E. coli* ML-35p were analyzed by Tricine sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (reducing conditions [30])

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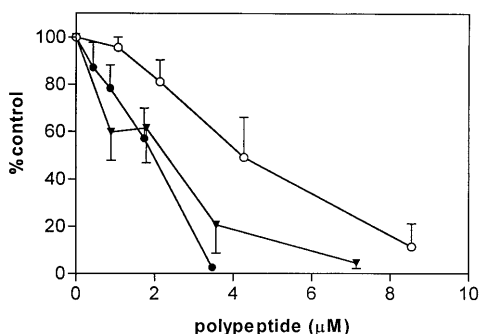


FIG. 1. Antibacterial activity of ALP, lysozyme, and defensins against *E. coli* ML-35p. Log-phase bacteria were incubated for 2 h at 37°C with the indicated concentrations of ALP (open circles), lysozyme (closed circles), or defensins (closed triangles), and the number of CFU was determined and used to calculate the percent control. The mean numbers of bacteria at the start and the end of the incubation were  $(5.4 \pm 1.7) \times 10^4$  and  $(81.7 \pm 46.4) \times 10^4$  CFU/ml, respectively. Data are presented as mean  $\pm$  SEM of four separate experiments.

and acid urea-PAGE (20); in addition, their reactivity with a rabbit antiserum raised against human defensins (a generous gift from T. Ganz, UCLA School of Medicine) was determined. This analysis demonstrated that these fractions consisted of a mixture of the human defensins HNP-1, HNP-2, and HNP-3.

Human leukocyte elastase was isolated from purulent sputum as previously described (8, 15), and lysozyme from human colostrum was purchased from Sigma (St. Louis, Mo.).

**Inhibitory activity of intact ALP and its domains against human leukocyte elastase.** Increasing amounts of ALP and its fragments were incubated with 8 nM elastase in 0.1 M Tris-HCl-0.2 M NaCl-0.05% (vol/vol) Triton X-100 (pH 7.8) for 15 min at 25°C. Residual elastase activity was determined by using 0.33 mM methoxysuccinyl-alanyl-alanyl-prolyl-valine *p*-nitroanilide, and hydrolysis of this substrate was monitored at 405 nm. Kinetic constants were calculated as described previously (1).

**Antibacterial assays.** The antibacterial activity of the proteins was investigated by using logarithmic-phase *E. coli* ML-35p (18) and *Staphylococcus aureus* 42D (kindly provided by R. van Furth, Leiden University Hospital, Leiden, The Netherlands) (21). To obtain bacteria in the mid-logarithmic phase, 50 to 200 µl of an overnight culture made in tryptic soy broth (TSB; Difco Laboratories, Detroit, Mich.) was added to 50 ml of TSB and incubated for 2.5 h at 37°C with shaking. The bacteria were then washed in 10 mM sodium phosphate buffer (NAPB; pH 7.4), and their concentration was estimated by spectrophotometry at  $A_{620}$  on the basis of the relationship  $A_{620} 0.2 = 5 \times 10^7$ /ml. The proteins that had been dialyzed against 0.01% (vol/vol) acetic acid were dried by vacuum centrifugation (Speed-Vac) in the tube that was used for the assay, and 100 µl of log-phase bacteria at  $5 \times 10^7$ /ml in 10 mM NAPB (pH 7.4) containing 1% (vol/vol) TSB was added. At the start of the incubation and after 2 h of incubation at 37°C, the number of CFU was determined by plating serial dilutions. All negative cultures were assigned the value 100 CFU/ml, which was the lowest value that could be detected by this procedure. The percentage control CFU was calculated by the formula  $N_{\text{exp}}/N_{\text{control}} \times 100$ , in which  $N_{\text{exp}}$  or  $N_{\text{control}}$  is the number of bacteria obtained after a 2-h incubation in the presence or absence, respectively, of the polypeptides. All incubations were performed in duplicate.

**Other methods.** Protein sequence alignments and charge analysis of the isolated ALP domains were obtained by using the PC/GENE and GeneWorks programs (Intelligenetics, Mountain View, Calif.).

**Analysis.** Data from the antibacterial assays are expressed in percent control CFU as mean  $\pm$  standard error of the mean (SEM). Statistical analysis of the data was performed with the GraphPad Instat program (GraphPad Software, San Diego, Calif.). Results from individual antibacterial assays were used to construct point-to-point curves from which the concentration of peptide causing 50% decrease in CFU (50% inhibitory concentration) for each of the individual experiments was calculated. This 50% inhibitory concentration was expressed as mean (95% confidence interval). Differences among treatment groups were examined by using a one-way analysis of variance followed by a Student-Newman-Keuls multiple-comparisons test. Differences were considered to be statistically significant at *P* values less than 0.05.

## RESULTS

Incubation of ALP with *E. coli* resulted in a marked decrease in the number of CFU (Fig. 1). Comparison of the 50% inhibitory concentration for each of the polypeptides revealed that the mean value of ALP was higher than that of defensins

and lysozyme (*P* < 0.05) (50% inhibitory concentrations [95% confidence intervals]: ALP, 4.7 µM [1.0 to 8.4]; lysozyme 1.8 µM [0.9 to 2.8]; and defensins, 1.4 µM [-0.7 to 3.5]). Microscopic evaluation indicated that the decrease in CFU in mixtures containing ALP was not likely caused by clumping of bacteria. The antibacterial activity of ALP was blocked by the presence of 0.15 M NaCl in the assay buffer (data not shown).

To investigate in which part of the ALP molecule this antibacterial activity is located, ALP was cleaved by mild acid treatment, resulting in the formation of two major fragments representing the first NH<sub>2</sub>-terminal (residues Ser-1 to Asp-49) and second COOH-terminal (residues Pro-50 to Ala-107) domains of ALP (37). Inhibition experiments using elastase demonstrated that all elastase inhibitory activity was present in the second domain (data not shown).

Next, intact ALP and the isolated domains were incubated in various concentrations with *E. coli* and *S. aureus* to compare their activities on a molar basis. Intact ALP and its first domain displayed antibacterial activity against *E. coli*, whereas the second domain was much less active (Fig. 2A). Comparison of the 50% inhibitory concentrations for each of the ALP preparations demonstrated that both intact ALP and the first domain were significantly more active against *E. coli* than the second domain (*P* < 0.01 and < 0.05, respectively) (50% inhibitory concentrations [95% confidence intervals]: ALP, 4.2 µM [1.3 to 7.0]; first domain, 12.1 µM [0.5 to 23.7]; and second domain, 23.7 µM [9.4 to 38.0]). It must be noted that in one of the four

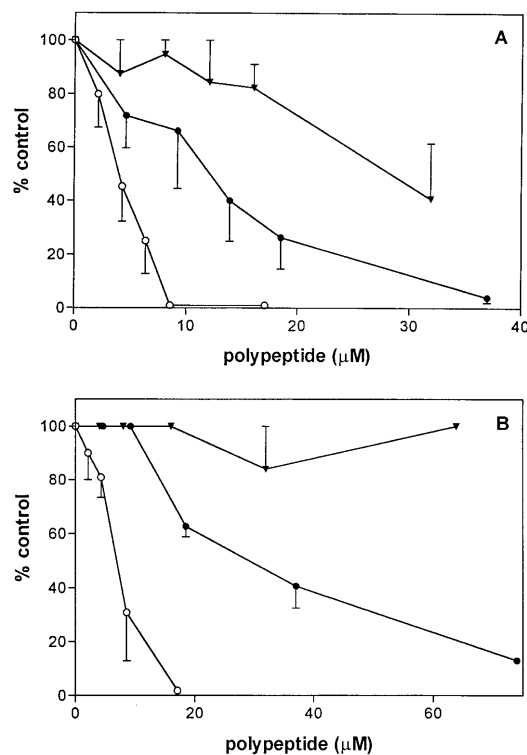


FIG. 2. Antibacterial activity of ALP and its isolated first and second domains against *E. coli* (A) and *S. aureus* (B). Log-phase bacteria were incubated for 2 h at 37°C with the indicated concentrations of intact ALP ( $M_r$ , 11,700; open circles) and its first ( $M_r$ , 5,400; closed circles) and second ( $M_r$ , 6,300; closed triangles) domains, and the number of CFU was determined and used to calculate the percent control. The mean numbers of bacteria at the start and the end of the incubation were  $(5.9 \pm 2.2) \times 10^4$  and  $(71.7 \pm 21.0) \times 10^4$  CFU/ml, respectively for *E. coli*; for *S. aureus*,  $(16 \pm 3.1) \times 10^4$  and  $(40.7 \pm 7.9) \times 10^4$  CFU/ml were present at the start and the end of the incubation. Data are presented as mean  $\pm$  SEM of four (A) or three (B) separate experiments.

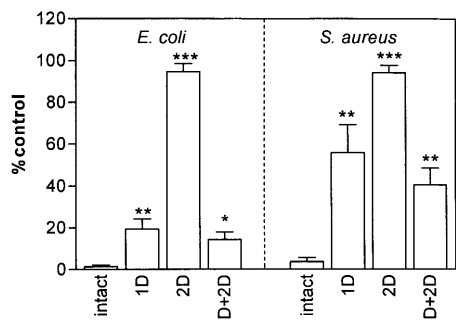


FIG. 3. Effect of intact ALP and its isolated domains on *E. coli* and *S. aureus*. Log-phase bacteria were incubated with 10  $\mu$ M intact ALP, its isolated first (1D) and second (2D) domains, and the combination of the two domains (1D + 2D), each at 10  $\mu$ M. After 2 h of incubation at 37°C, the number of CFU was determined and used to calculate the percent control. The mean numbers of bacteria at the start and the end of the incubation were  $(4.5 \pm 1.2) \times 10^4$  and  $(97.2 \pm 32.5) \times 10^4$  CFU/ml, respectively, for *E. coli*; for *S. aureus*,  $(3.6 \pm 0.2) \times 10^4$  and  $(16.4 \pm 1.3) \times 10^4$  CFU/ml were present at the start and the end of the incubation. Data are presented as mean  $\pm$  SEM of four (*E. coli*) or three (*S. aureus*) separate experiments. \*,  $P < 0.05$  versus intact ALP; \*\*,  $P < 0.01$  versus intact ALP; \*\*\*,  $P < 0.001$  versus intact ALP.

experiments it was not possible to calculate a 50% inhibitory concentration for the second domain because of lack of activity. Therefore, the value for this experiment was arbitrarily set at the highest concentration tested (31.9  $\mu$ M). The apparent difference in 50% inhibitory activity between ALP and its first domain did not reach statistical significance. Using *S. aureus* as the target microorganism, the second domain was even less active (Fig. 2B). Therefore, it was not possible to calculate a 50% inhibitory concentration. The difference between these values for intact ALP and its first domain did not reach statistical significance (50% inhibitory concentrations [95% confidence intervals]: intact ALP, 7.6  $\mu$ M (0.9 to 14.4); and first domain, 31.2  $\mu$ M [7.6 to 54.8]).

These experiments also revealed that the effect of ALP on *E. coli* is bactericidal, since after incubation with the highest concentration of intact ALP (17.1  $\mu$ M) for 2 h at 37°C, no culturable bacteria were recovered (<100 CFU/ml, on the basis of the detection limit of the assay), whereas at the start of the incubation,  $(5.9 \pm 2.2) \times 10^4$  CFU/ml was present. The effect of ALP on *S. aureus* is also bactericidal, since after 2 h in presence of 17.1  $\mu$ M intact ALP, significantly fewer bacteria were present than at the start of the incubation [ $(0.4 \pm 0.4) \times 10^4$  and  $(16 \pm 3.1) \times 10^4$  CFU/ml, respectively;  $P < 0.05$ , paired Student's *t* test].

In a subsequent set of experiments, *E. coli* and *S. aureus* were incubated with a fixed concentration of intact ALP, the isolated first and second domains, and the combination of the first and second domains (Fig. 3). Both isolated domains, alone or in combination, were significantly less active than intact ALP against either *E. coli* or *S. aureus*. The first domain was significantly more active than the second domain; the second domain did not display significant antibacterial activity at this concentration. Although the combined domains appeared to be a little more active than the isolated first domain, this difference did not reach statistical significance.

The two isolated domains were subjected to SDS-PAGE and acid urea-PAGE analysis (Fig. 4). Whereas both domains migrated similarly on SDS-PAGE, on acid urea-PAGE, the electrophoretic mobility of the first domain was higher than that of the second domain. This result, indicating that the first domain has a higher cationic charge than the second domain, was confirmed by estimation of the charge of domain 1 and 2 at pH

7, showing that the net charge of domain 1 (+7) was higher than that of domain 2 (+5). In addition, the charge profile showed a clustering of positive charge in the NH<sub>2</sub>-terminal half of domain 1 (data not shown).

## DISCUSSION

The main function of ALP is most likely to provide tissues protection against degradation by serine proteinases that are released from neutrophils during inflammation. The results from this study demonstrate that ALP also displays antibacterial activity: at concentrations that are found in various external secretions (10), ALP caused marked killing of both gram-negative (*E. coli*) and gram-positive (*S. aureus*) bacteria. Using isolated domains that were obtained after acid treatment of ALP, we found that the antibacterial activity of ALP is located in the first NH<sub>2</sub>-terminal domain of ALP. Previous studies (7, 16, 26, 37) and this study demonstrated that the proteinase inhibitory activity is located in the second, COOH-terminal domain. This finding indicates that the antibacterial and proteinase inhibitory activities of ALP are represented by different domains. We observed that on a molar basis, the antibacterial activity of the first ALP domain is lower than that of the intact molecule. Combination of the two isolated domains did not reconstitute the full activity of the intact ALP molecule. This may in part be explained by a possible (conformational) change in the first domain induced by the cleavage procedure of native ALP; alternatively, it may indicate that the second domain plays a role in the antibacterial activity in the intact molecule. Although the two ALP domains are homologous to each other, they apparently exert different functions. Analysis of the isolated domains by acid urea-PAGE demonstrated that the first domain had a higher electrophoretic mobility than the second domain, indicating a more cationic character as previously suggested (37). This finding, which was supported by the results of computer-assisted charge analysis of the protein sequence, may in part explain why the first domain displays antibacterial activity whereas the second domain is largely devoid of such activity. The possible involvement of the cationic properties in the activity of ALP is also supported by our observation that higher salt concentrations inhibit the antibacterial activity of ALP. Inhibition of antimicrobial activity at higher ionic strength has been reported for a variety of other antimicrobial proteins (19, 22). Although ionic interactions may be involved, the mechanism whereby ALP causes killing of bacteria is not yet clear. It has previously been reported that recombinant ALP is toxic to *E. coli* when expressed in this bacterium in high amounts (27). This toxicity was found to be due to its ability to bind to mRNA and DNA, resulting mainly

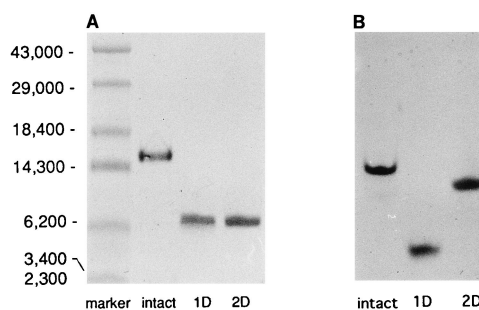


FIG. 4. Analysis of the isolated ALP domains by SDS-PAGE and acid urea-PAGE. Five micrograms of intact ALP and of first (1D) and second (2D) domains were subjected to Tricine SDS-PAGE (A) and acid urea-PAGE (B) and stained with Coomassie brilliant blue.  $M_r$ 's are indicated on the left.

in translation inhibition. Since in our study ALP was added exogenously to *E. coli*, such a mechanism alone cannot explain the observed antibacterial activity.

Antimicrobial properties of proteinase inhibitors have been previously reported. The cationic proteinase inhibitor aprotinin, present in bovine lung and other organs, displays antibacterial activity against both gram-positive and gram-negative bacteria (28). This activity of aprotinin did not require its proteinase inhibitory activity and was shown to be probably related to its cationic properties. In addition, a synthetic peptide mimicking the cysteine proteinase inhibitory site of human cystatin C was found to kill group A streptococci (2). Another example of an antimicrobial proteinase inhibitor is the aforementioned eNAP-2, a cationic polypeptide purified from equine neutrophils having limited sequence homology with ALP (3, 4). Both ALP and eNAP-2 contain a pattern of cysteine residues that is characteristic for members of the FDC family of proteins (3, 33). Alignment of the protein sequences of ALP and eNAP-2 demonstrated that most of the homology was found in the second proteinase inhibitory domain, not in the first antibacterial domain, of ALP.

Previous studies have suggested a role for human serine proteinase inhibitors in host defense against infections; these studies demonstrated that proteinase inhibitors are able to inhibit the elastase-mediated degradation of opsonins and receptors involved in phagocytosis (23, 36). A recent study in which saliva components exhibiting anti-human immunodeficiency virus type 1 activity were investigated identified ALP as the major active component (25). This antiviral activity of ALP is most likely not due to a direct interaction with the virus but was suggested to be the result of an interaction with the host cell. The results from the present study show that ALP may act directly as an antibacterial agent. Thus, ALP may be an addition to the array of locally produced antimicrobial polypeptides that have been identified in secretions and that include lysozyme, lactoferrin, and tracheal antimicrobial peptide (5, 34). Interestingly, the distribution of ALP in human tissues is much like that of another antimicrobial polypeptide, lysozyme (9, 12). In addition to locally produced antimicrobial polypeptides, especially purulent secretions may contain various antimicrobial polypeptides that are derived from neutrophils, including defensins.

At present, ALP is being considered as a therapeutic agent for treatment of inflammatory lung disease. Recombinant ALP has been administered to cystic fibrosis patients in a short-term study (24), but its clinical efficacy remains to be established. Since such diseases are often associated with (recurrent) bacterial infections, ALP may not only aid in restoring the balance between proteinases and proteinase inhibitors but also act as an antimicrobial agent.

In summary, the results from this study show that intact ALP and its isolated first NH<sub>2</sub>-terminal domain efficiently kill bacteria in vitro. This finding indicates that ALP may contribute to host defense against infection through its antibacterial activity. Additional studies will be required to determine whether ALP also kills lung pathogens.

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