Inactivation of Human α -1-Antitrypsin by a Tissue-destructive Protease of *Legionella pneumophila*

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Three extracellular proteases produced by *Legionella pneumophila* during growth in liquid medium were examined for their effects on human α -1-antitrypsin (α -1-AT). One of these proteases, tissue-destructive protease (TDP) destroyed completely the trypsin-inhibitory capacity of α -1-AT at protease:inhibitor molar ratios down to 0.002:1. After inactivation by TDP, the M_r of α -1-AT was reduced by 5000 in SDS-PAGE. This suggested that inactivation entailed only limited cleavage.

INTRODUCTION

α -1-Antitrypsin (α -1-AT) is the major protease inhibitor found in human serum (Travis & Johnson, 1981). Human α -1-AT is a specific inhibitor of serine proteases, including leucocyte elastase, pancreatic trypsin and chymotrypsin, cathepsin G, collagen and kallikrein. The main role of α -1-AT is the control of tissue destruction by these endogenous serine proteases.

Pseudomonas aeruginosa (Moriyama *et al.*, 1979) and *Serratia marcescens* (Molla *et al.*, 1986) elaborate non-serine metalloproteases which are capable of inactivating α -1-AT by specific cleavage. It has been postulated that this provides a mechanism which may contribute to the pathogenesis of the diseases, including bronchopneumonias, caused by these organisms.

A number of extracellular proteolytic activities have been described for *Legionella pneumophila*. In particular, Müller (1983) has described proteolytic activity against serum proteins by *L. pneumophila* when this organism was cultured on medium containing human serum. Müller demonstrated degradation of α -1-AT by a change in electrophoretic mobility using an immunoelectrophoretic technique.

Recently, six discrete extracellular proteolytic activities were isolated from *L. pneumophila* grown in broth culture (Conlan *et al.*, 1986). In the present study a lysine aminopeptidase (lys-AP), a leucine-AP and a tissue-destructive protease (TDP) of *L. pneumophila*, chosen on the basis of their relative abundance in culture supernates, were examined for their effect on α -1-AT.

METHODS

Enzymes. *L. pneumophila* proteases were obtained as described below. leu-AP and lys-AP were assayed using leucine *p*-nitroanilide (leu-*p*NA) and lysine-*p*NA substrates (Sigma), respectively. These were dissolved to 1 mM in 50 mM-Tris/HCl pH 7.0 (buffer A) and stored at 4 °C. For quantification of enzyme activity, 50 μ l of enzyme was added to 2.5 ml substrate (at 37 °C) in a 1 cm cuvette. Using heat-inactivated enzyme as a blank, increase in absorbance at 405 nm with time was measured in a spectrophotometer (Philips PU8720) with a sample compartment at 37 °C. TDP activity was quantified using hide powder azure (Calbiochem) as substrate. TDP (50 μ l; diluted in buffer A) was added to a 5 ml suspension of hide powder (10 mg ml⁻¹ in buffer A) which had been pre-warmed to 37 °C. Incubation at 37 °C was continued for 20 min and the reaction stopped by placing on

Abbreviations: α -1-AT, α -1-antitrypsin; TDP, tissue-destructive protease; AP, aminopeptidase; *p*NA, *p*-nitroanilide; BAPNA, *N* α -benzoyl-DL-arginine *p*-nitroanilide; FPLC, fast protein liquid chromatography.

ice. The remaining particulate substrate was removed by filtration (Whatman no. 1 filter) and absorbance of the coloured filtrate read at 595 nm in a 1 cm cuvette.

Bovine trypsin (Sigma type 1) was prepared as a 5 mg ml⁻¹ stock solution in 1 mM-HCl and stored at 4 °C. Trypsin substrate was *N*α-benzoyl-DL-arginine *p*-nitroanilide (BAPNA). This was dissolved in dimethyl sulphoxide and diluted to 1 mM in 100 mM-Tris/HCl, 10 mM-CaCl₂, pH 7.8 (buffer B). Trypsin activity was determined as for leu-AP above.

Enzyme units. One unit of enzyme activity was arbitrarily defined as the amount of enzyme which, in the appropriate assay (above), caused an increase in absorbance of 0.01 min⁻¹ at 37 °C. Protein was determined by absorbance at 280 nm. On this basis the specific activities [units (mg protein)⁻¹] of the enzymes used in the present investigation were determined to be as follows: trypsin (525), TDP (180), leu-AP (5800), lys-AP (8300).

Organism. Corby strain *Legionella pneumophila* was a serogroup 1 human isolate, kindly provided by Dr R. A. Swann, John Radcliffe Hospital, Oxford, UK. This organism had been passaged four times on charcoal yeast extract (CYE) agar (Edelstein, 1981) before use in the present study.

Preparation of *L. pneumophila* proteases. Concentrated culture supernatant fractions of *L. pneumophila* grown in yeast extract broth (YEB; Ristroph *et al.*, 1980) were prepared as previously described (Conlan *et al.*, 1986) except that the yeast extract component of the medium was ultrafiltered through a YM10 membrane (Amicon; *M_r* cut-off > 10000) to remove high-*M_r* material before inoculation. It has been reported that this procedure facilitates the purification of extracellular proteases of *L. pneumophila* (Dreyfus & Iglewski, 1986). Individual proteases present in concentrated culture supernates (10 ml of 100-fold concentrated supernate) were separated by Sephadex G100 gel-filtration and DEAE-cellulose ion-exchange chromatography as previously described (Conlan *et al.*, 1986). Proteases separated by ion-exchange chromatography on DEAE-cellulose were further purified by fast protein liquid chromatography (FPLC) as follows. Samples (10 ml) of individual proteases were mixed with 40 ml buffer A and applied to a 1 ml Mono Q (Pharmacia) anion-exchange column using a 50 ml sample loop. The sample was eluted with a linear gradient of NaCl (0–0.5 M, generated over 50 ml) in buffer A at a flow rate of 2 ml min⁻¹ and 1 ml fractions collected. Individual fractions were monitored for protease activity using specific substrates (Conlan *et al.*, 1986). FPLC fractionated proteases were stored at –70 °C, without further treatment, until required.

Degradation of α-1-AT by *L. pneumophila* proteases. Human α-1-AT was from Sigma. For these experiments the following *M_r* values were assumed: trypsin and α-1-AT, *M_r* 25000 and 58000 respectively (Moriyama *et al.*, 1979); *L. pneumophila* lys-AP, leu-AP and TDP, *M_r* 34000, 35000 and 40000 respectively (by SDS-PAGE). Individual *Legionella* proteases were mixed in a molar ratio enzyme:α-1-AT of 0.1, 0.1:1, 0.02:1, 0.002:1 and 0.001:1. The reaction mixture, which consisted of 10 µl of enzyme and 15 µl (8 µg) α-1-AT in buffer A, was incubated for 30 min at 37 °C. Trypsin (4 µg in 25 µl buffer B) was then added to the mixture and the incubation continued for 10 min. BAPNA (2.5 ml) was then added and the mixture was incubated for a further 10 min. The reaction was then stopped by placing the mixture on ice, the absorbance at 405 nm determined and the remaining trypsin-inhibitory capacity calculated. Controls were α-1-AT pre-incubated with buffer A or heat-inactivated *Legionella* protease (100 °C for 5 min) prior to addition of trypsin. Preliminary experiments demonstrated no activity of *Legionella* proteases against BAPNA. Pre-incubation of trypsin with *Legionella* proteases did not affect its activity against BAPNA.

Effect of TDP on pre-formed trypsin-α-1-AT complex. α-1-AT (8 µg in 15 µl buffer B) was mixed with 4 µg trypsin in 25 µl of the same buffer and incubated at 37 °C. After 10 min, 10 µl TDP (55 µg ml⁻¹) was added to the reaction mixture and the incubation continued for 30 min. Residual trypsin activity was determined by addition of 2.5 ml BAPNA for a further 10 min, as above. Controls were run in which TDP was replaced either by heat-inactivated TDP or buffer A, or α-1-AT was replaced by buffer B.

SDS-PAGE analysis of inactivation of α-1-AT by TDP. TDP was mixed with α-1-AT at a ratio of 0.02:1 and the mixture was incubated for 30 min at 37 °C. The reaction mixture was then mixed with an equal volume of sample buffer (10%, v/v, glycerol, 2%, w/v, SDS, 5%, v/v, mercaptoethanol, 0.001%, w/v, bromophenol blue in 62.5 mM-Tris/HCl, pH 6.8) and boiled for 5 min. Samples and *M_r* markers (Sigma) were separated by SDS-PAGE, incorporating a 12% (w/v) separating gel, using the Laemmli buffer system (Laemmli, 1970). Electrophoresis was for 20 h at 8 mA constant current, after which gels were stained with Coomassie brilliant blue.

Effect of TDP on trypsin-inhibitory capacity of serum. Human serum was obtained from healthy volunteers and stored at –70 °C as 1 ml samples until required. Serum α-1-AT content was determined using a commercial radial immunodiffusion assay (Partigen; Behringwerke). Doubling dilution series of thawed sera (25 µl through 25 µl volumes buffer B) were made in 96-well microtitration plates. TDP (25 µl at 55 µg ml⁻¹) was then added to each well and the plates were incubated for 30 min. Trypsin (50 µl of a 1 in 40 dilution of stock in buffer A) was added to each well and incubation continued for 30 min. Remaining trypsin-inhibitory capacity was measured by adding 150 µl BAPNA per well, incubating plates for a further 10 min and reading them in a photometer (Titertek Multiscan) fitted with a filter at 405 nm. Controls, which consisted of heat-inactivated TDP, were included for each serum. The effect of TDP on trypsin-inhibitory capacity was further examined by fractionating TDP-treated

serum by FPLC. Serum (250 μ l of a 1 in 4 dilution in buffer A) was incubated with 250 μ l TDP (55 μ g ml⁻¹). After 30 min at 37 °C the reaction mixture was diluted with an equal volume of 25 mM-Tris/HCl pH 8.5 (buffer C) and 500 μ l was separated by ion-exchange using a Mono Q column as described below.

Fractionation of human sera by FPLC. Neat serum (50 μ l) was diluted 1 in 20 in buffer C and a 500 μ l sample was applied to a 1 ml Mono Q column. The sample was eluted with a linear gradient of NaCl (0–0.5 M in buffer C generated over 40 ml) at a flow rate of 2 ml min⁻¹ and 1 ml fractions were collected. Human α -1-AT and α -2-macroglobulin (Sigma) were run separately by the same method as standards. Anti-trypsin activity of FPLC fractions was determined in 96-well plates as follows. Each serum fraction (50 μ l) was mixed with 50 μ l trypsin (1 in 400 dilution of stock in buffer A) and incubated for 30 min at 37 °C. After addition of 150 μ l BAPNA to each well, plates were incubated for a further 10 min and absorbance at 405 nm was measured. Inhibitory activity of serum fractions for leu-AP and lys-AP was similarly determined using specific substrates and 1 in 20 dilutions of stock enzymes. The uneven size of hide powder particles rendered this substrate unsuitable for use in plate assays for TDP activity. Therefore, Azocoll (Sigma) substrate was used instead. This insoluble substrate was prepared in buffer A (10 mg ml⁻¹) and kept in suspension by stirring during dispensing. Following incubation of serum fractions with TDP (1 in 20 dilution of 55 μ g ml⁻¹ frozen stock in buffer A), 100 μ l volumes of the reaction mixture were transferred to the corresponding wells of a second plate which contained 150 μ l Azocoll substrate per well. The second plate was incubated for 30 min at 37 °C, then 200 μ l volumes were carefully withdrawn to a third plate, avoiding disturbance of remaining sedimented substrate, and this plate was read at 492 nm.

RESULTS

Trypsin-inhibitory capacity of serum fractions separated by FPLC

Serum fractionated by FPLC, using a Mono Q anion-exchange column, produced a complex profile of absorbance at 280 nm (Fig. 1a). Trypsin inhibition assays demonstrated that inhibitory activity (Fig. 1b) co-eluted with a peak of absorbance at 280 nm (Fig. 1a). This peak eluted in the same volume as purified human α -1-AT (Fig. 1a). None of the *L. pneumophila* proteases were inhibited by this material. α -2-Macroglobulin, another serum protease inhibitor, was not associated with α -1-AT but eluted in later fractions which also did not inhibit any of the test proteases. The peaks and troughs of proteolytic activity observed in regions of the profile remote from fractions containing α -1-AT activity were also seen with blank NaCl gradients. The separation and trypsin-inhibition profiles were very reproducible and gave identical results for each of four test sera.

*Effect of *L. pneumophila* proteases on inhibitory capacity of α -1-AT for trypsin*

When purified α -1-AT was pre-incubated with the *L. pneumophila* proteases TDP, leu-AP and lys-AP, only TDP had an effect on the inhibitory capacity of α -1-AT for trypsin under the assay conditions used. Because of this finding all further experimentation was confined to TDP. At TDP:inhibitor molar ratios down to 0.002:1 there was complete destruction of trypsin-inhibitory capacity; even at the lower TDP:inhibitor ratio 0.001:1, α -1-AT retained only 25% of initial inhibitory capacity.

SDS-PAGE analysis of α -1-AT inactivation by TDP

SDS-PAGE was used to determine the nature of the inactivation of α -1-AT by TDP of *L. pneumophila*. Inactivation of α -1-AT gave rise to a single polypeptide band with an M_r 5000 lower than that of the active inhibitor (Fig. 2).

Stability of α -1-AT-trypsin complex in the presence of TDP

The complex formed between α -1-AT and trypsin in the absence of TDP was stable upon incubation with the latter; trypsin activity was not regenerated in the presence of TDP.

Effect of TDP on trypsin-inhibitory capacity of serum

The levels of α -1-AT present in the sera used in these experiments were within the range 2.48–2.72 mg ml⁻¹. *L. pneumophila* TDP caused destruction of α -1-AT present in normal human serum. A marked decrease in the trypsin-inhibitory capacity of whole serum was observed following incubation with TDP. Total inactivation of trypsin-inhibitory capacity occurred at TDP: α -1-AT ratios above 0.1:1. This decrease in trypsin-inhibitory capacity was of a similar

Fig. 1. Anti-protease activity of normal human serum. (a) Whole human serum was fractionated by FPLC on a Mono Q column according to Methods. Standards (broken peaks) were α -1-AT (1) and α -2-macroglobulin (2). ---, NaCl gradient. (b) Individual serum fractions were assayed for protease inhibitory capacity against trypsin ($\blacktriangle$), leu-AP ($\bullet$), lys-AP ($\blacksquare$) and TDP ($\circ$).

order for each of four test sera. No decrease in α -1-AT, measured as antigen by radial immunodiffusion in gel, was observed in sera treated with TDP.

When TDP-treated serum was fractionated by FPLC, loss of trypsin-inhibitory capacity was accompanied by partial loss of the peak of absorbance at 280 nm associated with α -1-AT (Fig. 3b, c). The protein profiles obtained for serum treated with either TDP or heat-inactivated (100 °C for 5 min) TDP (Fig. 3a, b) exhibited a peak at the start of the gradient which represented a shift in the profile obtained with untreated serum (Fig. 1a). This shift was probably due to the higher salt concentration of the TDP-treated samples; the TDP eluted from Mono Q in 0.4 M-NaCl and was used without further treatment to avoid loss of activity.

DISCUSSION

Three extracellular proteases purified from broth culture supernates of *L. pneumophila* were examined for their effects on the serine protease inhibitor α -1-AT. None of the *Legionella* proteases were inhibited by α -1-AT; these proteases are resistant to inhibition by phenylmethylsulphonyl fluoride (Conlan, 1987; S. Simpson, personal communication) which is a specific inhibitor of serine proteases. One of the *Legionella* proteases, TDP, destroyed completely the trypsin-inhibitory capacity of purified α -1-AT at low protease:inhibitor molar ratios. The M_r of α -1-AT treated with TDP was 5000 lower than that of native α -1-AT, a decrease

Fig. 2. SDS-PAGE analysis of α -1-AT inactivated by reaction with *L. pneumophila* TDP. Lane 1, M_r markers; lane 2, α -1-AT incubated with TDP for 30 min at 37 °C at a protease:inhibitor molar ratio of 0.02:1; lane 3, α -1-AT incubated as above in the absence of TDP; lane 4, TDP at the test concentration.

identical to that observed with α -1-AT treated with *P. aeruginosa* elastase (Moriyama *et al.*, 1979) and *S. marcescens* metalloprotease (Molla *et al.*, 1986). This uniform decrease in M_r indicates that α -1-AT is inactivated by proteolysis at a specific cleavage point. Also, TDP inactivated α -1-AT present in normal human serum, although only at higher molar ratios of protease:inhibitor than those effective with purified α -1-AT. This could be due to other serum proteins acting as substrate for TDP in competition with α -1-AT. Once trypsin was bound by α -1-AT, the resultant complex was stable to treatment with TDP. Thus, to exert an effect *in vivo*, TDP would have to inactivate α -1-AT before the inhibitor could complex with serine proteases.

Fig. 3. FPLC analysis of the effect of TDP on the trypsin-inhibitory capacity of normal human serum. (a) Protein profile of serum treated with heat-inactivated TDP and fractionated by FPLC. ---, NaCl gradient. (b) Protein profile of serum treated with active TDP and fractionated by FPLC. Broken peak, α -1-AT standard; ---, NaCl gradient. (c) Inhibitory capacity for trypsin of serum fractions following treatment of whole sera with TDP (■) or heat-inactivated TDP (▲) was determined as described in Methods.

TDP causes pulmonary lesions similar to those observed in human and experimental Legionnaires' disease (Baskerville *et al.*, 1986; Conlan *et al.*, 1986). This protease has also been demonstrated in the lungs of guinea-pigs exposed to lethal aerosols of *L. pneumophila* (Williams *et al.*, 1987; Conlan, 1987). Furthermore, TDP was detected in the lungs of these experimental animals in amounts equivalent to the lethal dose of this purified protease administered by the intranasal route (Baskerville *et al.*, 1986). Against this background of massive tissue-destructive activity of the bacterial protease it is difficult to judge the relative importance of α -1-AT

inactivation as a pathogenetic mechanism in Legionnaires' disease. However, extensive necrosis of host inflammatory cells and alveolar epithelium is observed during the course of Legionnaires' disease (Blackmon *et al.*, 1981; Winn & Myerowitz, 1981). Host proteases released by this necrosis would have the potential to cause further pulmonary damage were they not inhibited by α -1-AT. Reduced levels of anti-protease activity have been demonstrated in the lungs of smokers (Gadek *et al.*, 1979) and in the lungs of patients, mainly elderly and with underlying disease, suffering from a variety of bacterial pneumonias (Abrams *et al.*, 1984). This may, in part, explain the increased susceptibility to Legionnaires' disease of these high-risk individuals.

The levels of α -1-AT present in the lungs of guinea-pigs with *L. pneumophila* infections should be quantified. With this information it may be possible to predict the extent of α -1-AT inactivation by the amounts of TDP known to be present in the lungs of these animals during the course of infection. The effects of *Legionella* proteases on other host proteins, of potential relevance to the pathogenesis of Legionnaires' disease, are currently under investigation.

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