BACTERIAL PATHOGENICITY

Differentiation of thermolysins and serralysins by monoclonal antibodies

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Two monoclonal antibodies (MAbs) to a 36-kDa extracellular metalloprotease (PSCP) from Burkholderia (Pseudomonas) cepacia were found to react with thermolysin, Pseudomonas aeruginosa elastase, alkaline protease (Apr) and LasA, Serratia marcescens protease (SMP), Aeromonas hydrophila protease (AhP), and both the lethal factor (LF) and protective antigen (PA) of Bacillus anthracis on immunoblots. The MAbs were capable of neutralising the proteolytic activity of thermolysin, P. aeruginosa elastase and PSCP but not that of Apr, SMP, and AhP. These results suggest that these MAbs may be able to differentiate between the thermolysin and serralysin family of metalloproteases on the basis of their neutralisation capability and could, therefore, be useful tools in the characterisation of new bacterial proteases.

Introduction

Many bacteria secrete extracellular proteases. Some of these proteases are toxins or factors involved in virulence, whereas others degrade proteins to produce small peptides and amino acids which can be utilised by the bacterium. Bacteria synthesise numerous proteases belonging to the serine-, cysteine- and metalloprotease classes [1]. Bacterial metalloproteases can be classified in these groups: the thermolysins and serralysins. Proteases are generally classified in these groups on the basis of amino-acid sequence homology. The most thoroughly characterised metalloprotease is thermolysin [2], a heat-stable neutral metalloprotease produced by Bacillus therrnoproteolyticus. It contains the HEXXH zinc-binding motif which is characteristic of zinc metalloproteases [3]. Many other thermolysin- like bacterial metalloproteases have been identified. Legionella pneumophila secretes a 38-kDa zinc metalloprotease called the major secretory protein (Msp) that may play a role in the pathogenesis of L. pneumophila infection [4, 5]. Vibrio cholerae secretes a 33-kDa zinc metalloprotease (HA/protease) [6], which can cleave biologically important substrates such as mucin, fibronectin and bactoferritin [7]. Pseudomonas aeruginosa secretes two well-characterised metalloproteases, elastase [8] and alkaline protease [9, 10]. P. aeruginosa elastase has been shown to degrade or inactivate several biologically important substrates such as fibrin, collagen, complement, human immunoglobulin G (IgG), transferrin and lactoferrin [11, 12]. Elastase exhibits considerable amino-acid sequence homology to thermolysin, V. cholerae HA/protease, and Msp [2, 5, 8]. Elastase, HA/protease and Msp are all synthesised as large prepropeptides which are processed to a mature proteolytically active form [8, 13, 14].

The serralysin group of proteases includes P. aeruginosa alkaline protease and the extracellular metalloproteases from Serratia marcescens and Erwinia chrysanthemi [2, 9, 10]. These three metalloproteases lack typical signal sequences at their N-termini and are preceded by short propeptides [15]. The S. marcescens 50-kDa metalloprotease (SM protease) is thought to be an important virulence factor involved in both keratitis and pulmonary infections [16, 17]. E. chrysanthemi secretes at least four different metalloproteases [18]. E. chrysanthemi B protease shares 60% homology with the SM protease.

P. aeruginosa secretes another protease LasA, that is synthesised as a 42-kDa protein, which is processed to a 21-kDa active peptide. LasA has both elastolytic and staphyloptic activity [19, 20]. Zinc stimulates the production of LasA [21]; however, LasA activity is not inhibited by classic metalloprotease inhibitors such as EDTA [22]. Aeromonas hydrophila secretes a protease (AhP) which has an amino-terminal sequence 69% identical to LasA [23]. AhP is a 19-kDa metalloprotease that degrades fibrin.

Interestingly, it has been found that tetanus toxin and several of the botulinum toxins are metalloproteases [24–27]. Recently, Klimpel et al. [28] reported that the anthrax lethal toxin factor (LF) contains a zinc metalloprotease consensus sequence. LF may have
the third zinc ligand on the amino-terminal side of the consensus zinc metalloprotease site, making it more similar to *P. aeruginosa* alkaline protease and the SM protease than to the classical thermolysin-like proteases. However, all these zinc metalloproteases exhibit a great deal of homology especially around the zinc-binding motif [2].

*Burkholderia* (*Pseudomonas*) *cepacia* has been shown to secrete at least two proteases, a 36-kDa protease (PSCP) with high protease activity and a 40-kDa protease with low activity [29, 30]. PSCP was shown to be a metalloprotease that can degrade gelatin, collagen and hide powder azure but not human immunoglobulins [30]. Monoclonal antibodies (MAbs) to PSCP have been used to demonstrate that the *Bur. cepacia* protease was immunologically related to *P. aeruginosa* elastase and the *V. cholerae* HA/protease [29]. Furthermore, MAbs were isolated that could neutralise *P. aeruginosa* elastase and the *V. cholerae* HA/protease but not *P. aeruginosa* alkaline protease. Thus, it was thought that it was possible that these MAbs could distinguish between two distinct families of bacterial metalloproteases by their neutralisation capabilities. This study aimed to determine whether two representative MAbs to PSCP could recognise other metalloproteases such as thermolysin, the SM protease, AhP and LasA. Also, the ability of these MAbs to neutralise the activity of thermolysin-like proteases (e.g., *B. thermoproteolyticus* thermolysin, *P. aeruginosa* elastase, *V. cholerae* HA/protease) and members of the serralysin family of metalloproteases (e.g., the SM protease and *P. aeruginosa* alkaline protease) was compared.

**Materials and methods**

**Proteases**

Elastase and alkaline protease, prepared from *P. aeruginosa*, were purchased from Nagase Biochemical Inc. (Osaka, Japan). Thermolysin from *B. thermoproteolyticus* and the *Serratia* metalloprotease (SMP) from *S. marcescens* were purchased from Sigma. The *A. hydrophila* proteinase (AhP) was generously provided by Dr M. Wizorek (Cortech Inc., Denver, Colorado, USA). Dr E. Kessler (Tel-Aviv University Sakler Faculty of Medicine, Israel) kindly provided *P. aeruginosa* LasA. Anthrax toxin lethal factor and protective antigen were generously provided by Drs K. Klimpel and S. Leppla (NIH, Bethesda, MD, USA). *Bur. cepacia* PSCP and 40-kDa proteases were purified as previously described [29].

**Monoclonal antibodies**

The MAbs employed in this study have been described previously [29]. MAb production was based on the method of Kohler and Milstein [12]. BALB/c mice were given four intraperitoneal (i.p.) injections of 30 μg of PSCP purified from *Bur. cepacia* Pc715j on days 0, 7, 14 and 21. Three days after the final injection, spleens were removed and spleen cells were collected in a sterile petri dish (Nunc) containing DMEM. Spleen and NS-1 cells (10:1) were fused by polyethylene glycol 4000 (Merck) 50% w/v. Fused cells were dispersed into 24-well plates at 6 x 10⁶ input spleen cells/well. The hybridoma cell lines were maintained for 14 days in DMEM supplemented with penicillin, streptomycin, glutamine, calf supreme 10%, 100 mM hypoxanthine, 1 mM aminopterin and 16 mM thymidine. This medium was designated HAT. Hybridoma cell lines producing MAbs to PSCP, detectable by enzyme-linked immunosorbent assay (ELISA) or immunoblotting, or both, were transferred to hypoxanthine-thymidine (HT) medium and cloned three times by limiting dilution in 96-well plates (Nunc). Antibody-producing cells (2 x 10⁶) were injected into pristane-primed BALB/c mice for ascites production.

The isotype of the antibodies in the ascitic fluid was determined with a mouse typer subsotyping kit (BioRad Laboratories) as recommended by the manufacturer.

Five MAbs (36-1-5, 36-5-15, 36-6-6, 36-6-8 and 36-9-19), all of the IgM isotype, were identified which neutralised PSCP [29]; two (36-6-6 and 36-6-8) were chosen for further characterisation in this study. These antibodies were selected because of their high titre and strong reaction on immunoblots. For the neutralisation experiments, MAbs were purified with the Pierce IgM purification kit as recommended by the manufacturer. To determine the ability of these MAbs to react with other proteases, proteins (3-5 μg) were separated by SDS-PAGE [31] and transferred to immobilon membranes by the method of Towbin et al. [32]. After blocking with BSA 5%, the blot was incubated with MAb 36-6-6 (1 in 10000) or MAb 36-6-8 (1 in 500) in BSA 5% for 1 h at 37°C. After extensive washing, the blot was incubated with goat anti-mouse antibody-peroxidase conjugate (1 in 2000) for 1 h at 37°C. After washing, the blot was developed with horse-radish peroxidase [29].

**Protease assay**

Proteolytic activity was quantified by measuring the hydrolysis of hide powder azure (Sigma) in an assay based on the method of Rinderknecht et al. [33]. Proteases were mixed with 20 mg of hide powder azure in a final volume of 1.5 ml in 10 mM Tris, pH 8.0. The reaction mixtures were incubated for 2 h at 37°C with vigorous shaking. The samples were centrifuged at 6000 g for 10 min and the A₅₉₅ of the supernates was measured. To account for non-specific hydrolysis of the hide powder azure, control samples without protease were incubated in parallel and the A₅₉₅ of the resultant supernate was subtracted as background. Stocks (1 mg/ml) of each protease tested were prepared. Dilutions
were made and protease assays were performed as described above. Concentrations of protease that would give an $A_{595}$ of c. 1.0 were used in the neutralisation assays. The amounts used were SMP 0.4 µg, *P. aeruginosa* elastase 0.01 µg, thermolysin 0.05 µg, PSCP 0.5 µg, *P. aeruginosa* alkaline protease 0.2 µg and AhP 0.26 µg. These proteases were pre-incubated with either Tris-HCl buffer, control ascitic fluid (1 in 500) or MAb (1 in 500) in a final volume of 1.0 ml, for 2 h at 37°C. The samples were centrifuged briefly and the supernates were transferred to new tubes containing Tris-buffer 0.5 ml and hide powder azure 20 mg. Proteolytic activity was measured as described above.

**ELISA to measure antibody affinity**

An ELISA was performed to compare the affinity of MAb 36-6-6 for the various proteases. Immulon 4 96-well microtitration plates (Dynatech) were coated with 100 µl of each protease (1 µg/ml) in carbonate coating buffer (pH 9.6) for 2 h at 37°C. The plates were washed with buffer A (phosphate-buffered saline, pH 7.4, BSA 0.05% w/v) and blocked for 2 h at 37°C by the addition of 250 µl of PBS, BSA 5% to each well. After two washes with buffer A, serial two-fold dilutions starting at 1 in 200 of MAb 36-6-6 in buffer A (100 µl/well) were applied to the wells. All assays were performed in duplicate and included negative controls in which no antibody was present. After incubation for 2 h at 37°C, the plates were washed five times with buffer A. Goat anti-mouse peroxidase conjugate (100 µl, 1 in 2000; Kirkegaard and Perry Laboratories, Inc.) was added to each well and incubated at 37°C for 2 h. Wells were washed five times with buffer A and aspirated thoroughly. ABTS substrate (BioRad) 100 µl/well, was applied to each well and, after agitation at room temperature for 15 min, the $A_{405}$ was determined with an EL340 BioKinetics Reader (Bio-Tek Instruments).

**Results and discussion**

Previously, we reported that MAbs 36-6-6 and 36-6-8 reacted with *Bur. cepacia* PSCP and 40-kDa proteases, *P. aeruginosa* elastase, *P. aeruginosa* alkaline protease (Apr) and the *V. cholerae* HA/protease on immunoblots [29]. To determine the extent of cross-reactivity of these MAbs with metalloproteases, the ability of MAb 36-6-6 and 36-6-8 to react with other metalloproteases was examined on immunoblots. In addition to those proteases previously described, MAb 36-6-6 reacted with thermolysin (TLN) from *B. thermoproteolyticus*, *S. marcescens* protease (SMP), *P. aeruginosa* LasA and the *A. hydrophila* protease (AhP) (Fig. 1). The corresponding Coomassie blue-stained SDS-PAGE gel of the protease preparations used is shown in Fig. 2.

![Fig. 1. Immunoblot of proteases with MAb 36-6-6. Proteins (3–5 µg) were separated by SDS-PAGE and blotted on to immobilon. Lane 1, mol. wt markers, 2, *P. aeruginosa* Las A; 3, *B. thermoproteolyticus* thermolysin (TLN); 4, *A. hydrophila* protease (AhP); 5, PSCP; 6, *Bur. cepacia* 40-kDa protease; 7, *S. marcescens* major metalloprotease (SMP); 8, blank; 9, *P. aeruginosa* alkaline protease (Apr).]
Similar results were obtained with MAb 36-6-8 (data not shown). This result suggests that the epitope(s) recognised by these MAbs are conserved among all these metalloproteases. MAb 36-6-6 was shown previously not to react with chymotrypsin [29] which indicates that these antibodies do not recognise serine proteases. Anthrax lethal factor (LF) contains a zinc metalloprotease consensus sequence; however, no substrate for proteolytic activity has yet been described [28]. MAbs 36-6-6 and 36-6-8 also recognised anthrax LF weakly on immunoblots (Fig. 3 and data not shown). The faint reaction was detectable only after a significantly longer development time, even though an equivalent amount of LF was loaded on the gel as other proteins which reacted strongly, suggesting that either the site(s) recognised by these MAbs is not as well conserved in anthrax LF, or that SDS-PAGE affects the antigenic site. Curiously, MAb 36-6-6 reacted with the anthrax PA, which has not been reported to be a metalloprotease, more strongly than with the LF (Fig. 3). Comparison of the P. aeruginosa elastase and anthrax PA amino-acid sequences reveals very little homology. At present the reason for the cross-reactivity is unknown.

As MAbs 36-6-6 and 36-6-8 were shown previously to neutralise the activity of P. aeruginosa elastase, the V. cholerae HA/protease and PSCP but not Apr [29], we hypothesised that these MAbs would be able to distinguish between two families of metalloproteases [15,34], the thermolysins and the serralysins, by their neutralisation capabilities. MAbs 36-6-6 and 36-6-8 were found to neutralise elastase, the V. cholerae HA/protease and TLN but not Apr and SMP (Table 1). Therefore, although these metalloproteases have conserved regions which are recognised by these MAbs, in the case of the TLN-like metalloproteases antibody binding leads to neutralisation but, with the serralysins, binding of the MAb does not lead to neutralisation. The difference in the ability of these MAbs to neutralise may be due to differences in amino-acid composition of the epitope or slight changes in tertiary configuration. MAb 36-6-8 did not neutralise AhP activity (Table 1), suggesting that this protease may be more similar to SMP than to TLN and may be a member of the metzincin family of metalloproteases to which the serralysins belong [15]. LasA does not utilise hide powder azure as a substrate. It has been reported to cleave elastin, β-casein and to have staphylolytic activity [20,22]. Recently LasA has been shown not to be inhibited by EDTA and o-phenanthroline, and, therefore, may not be a metalloprotease [22], although zinc has been shown to enhance LasA activity [21]. A proteolytic substrate for LF has not yet been reported. Therefore, the ability of the MAbs to neutralise the activity of LF and LasA was not examined.

It is possible that differences in neutralisation may reflect a difference in affinity of the MAbs for the various proteases. Therefore, the affinity of MAb 36-6-8 for the proteases was estimated by comparing the dose-response curves of the MAb with the same concentration of each protease in an ELISA [35]. As shown in Fig. 4, there did not appear to be a correlation between the MAb binding curves for the
Differences in the ability of the MAbs to neutralise thermolysin and serralysin proteases do not appear to be due to marked differences in the affinity of the MAbs for these proteases.

Although the serralysins and thermolysins share a common zinc-binding motif (XXHEXXHX) and the upper domains are topologically similar, there are differences in the active sites [15]. For example, the active site helix of thermolysin extends beyond the HEXXH motif, with a polypeptide chain that loops back to the active site and is responsible for the third zinc-binding residue (E166). In the case of the metzincins (astacins, serralysins, snake venom and matrix metalloproteases), the zinc-binding motif has been extended to HEXXHXXGXXH. Thermolysins have a consensus sequence NEXXSD (E166 for TLN) that is not found in the serralysins. At this approximate region, the serralysins (and other metzincins) have a conserved methionine which is part of the methionine-containing turn, which may play a role in the structural integrity of the active site. This site is not present in the thermolysins. Thus, it is possible that a MAb may bind to a site near the active site of both thermolysins and serralysins, but only neutralise the members of the thermolysin family.

It is also possible that the epitope(s) recognised by these two MAbs are located near the active site in the thermolysin group of proteases but the MAbs recognize a different epitope in another region of the serralysin enzymes. These epitope(s) may have a similar amino-acid sequence to the epitope, which

**Table 1. Ability of MAbs to *Bur. cepacia* PSCP to neutralise metalloprotease activity**

<table>
<thead>
<tr>
<th>Protease</th>
<th>MAb 36-6-6</th>
<th>MAb 36-6-8</th>
<th>MCA-6†</th>
</tr>
</thead>
<tbody>
<tr>
<td>PSCP</td>
<td>0.3 (7)‡</td>
<td>11.8 (5)‡</td>
<td>75 (5)</td>
</tr>
<tr>
<td>Elastase</td>
<td>0 (1)‡</td>
<td>0 (3)‡</td>
<td>98 (5)</td>
</tr>
<tr>
<td>Thermolysin</td>
<td>3 (6)‡</td>
<td>0 (14)‡</td>
<td>87 (7)</td>
</tr>
<tr>
<td>Serratia protease</td>
<td>131 (9)</td>
<td>71 (15)</td>
<td>129 (8)</td>
</tr>
<tr>
<td>Alkaline protease</td>
<td>106 (2)</td>
<td>108 (5)</td>
<td>108 (2)</td>
</tr>
<tr>
<td>AhP</td>
<td>ND</td>
<td>137 (16)</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND, not done.

*Proteolytic activity was expressed as a percentage of the control (100%) which contained no antibody. Values represent mean (SD) of three replicates.
†MCA-6 is a purified antibody to *P. aeruginosa* FBP.
‡Significantly different (p < 0.001) than reactions containing no antibody with Bonferroni multiple comparisons test.
may be located near the active site of the thermolysins. Experiments are currently underway in this laboratory to map the epitopes recognised by the MAbs.

The finding that MAbs to the PSCP neutralise elastase, TLN, the V. cholerae HA/protease as well as PSCP but not Apr, SMP or AhP suggests that the PSCP belongs to the thermolysin metalloprotease family and that AhP may belong to the serralysin family of metalloproteases. It also appears that these MAbs may be able to differentiate between the serralysins and thermolysins on the basis of their neutralisation capability. Therefore, these antibodies may be useful in the initial classification of proteases when amino-acid sequence information may not be available.

This study was supported by the Canadian Bacterial Diseases Network of Centres of Excellence.

References

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