Proteus mirabilis strains of diverse type have IgA protease activity

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Summary. A strain of *Proteus mirabilis* associated with chronic urinary tract infection was found to produce an EDTA-sensitive IgA protease that cleaved the IgA heavy chain into two fragments at sites different from those attacked by other microbial IgA1 proteases. Another 14 *P. mirabilis* strains of diverse type and from various clinical conditions also produced a similar IgA protease. This enzyme may be a virulence determinant of *P. mirabilis*.

Introduction

Although infections of the urinary tract with *Proteus mirabilis* occur less frequently than those with *Escherichia coli* except in young boys (Bergström, 1972; Hallett *et al.*, 1976; Khan *et al.*, 1978) and the elderly (Walkey *et al.*, 1967; Senior, 1979), the consequences may be more serious. The predilection of *P. mirabilis* is for the upper urinary tract (Fairley *et al.*, 1971; Svandborg Edén *et al.*, 1980) where damage to the renal tubular epithelium (Braude *et al.*, 1960) and stone formation (Griffith *et al.*, 1973) may take place.

The virulence of *P. mirabilis* for the urinary tract may arise through the interplay of several factors including rapid growth in urine and the formation of potent urease isoenzymes which are important in human and experimental urinary tract infections (MacLaren, 1968 and 1969; Musher *et al.*, 1975; Senior *et al.*, 1980; Senior, 1983*a*). Motility also appears to be important in establishing ascending pyelonephritis (Pazin and Braude, 1974). *P. mirabilis* cells, unlike those of *E. coli*, have the ability to directly invade mammalian cells (Braude and Sieminski, 1960; Peerbooms *et al.*, 1984) possibly through the activity of a cell-bound haemolysin (Peerbooms *et al.*, 1983). The presence of certain fimbriae (Silverblatt, 1974; Silverblatt and Ofek, 1978), proticines and proticine receptors (Senior, 1979) may also be associated with *P. mirabilis* virulence but the relative importance of these factors is still unclear (Adegbola *et al.*, 1983; Senior, 1983*b*).

In this study the ability of *P. mirabilis* strains associated with urinary tract infections to produce

a new type of IgA protease was demonstrated. Production of this enzyme *in vivo* may enable *P. mirabilis* to overcome the antibody defence of the urinary tract mucosal surfaces resulting in the establishment of infection.

Materials and methods

Bacteria

Single test strains of *Haemophilus influenzae*, *Neisseria gonorrhoeae* and *Streptococcus pneumoniae* were originally isolated from clinical specimens and had been maintained for several months on cooked blood agar. Fifteen strains of *Proteus mirabilis* (see table) were selected from a wide variety of clinical sources and places and were of diverse bacteriocine (P/S) and O antigenic types. The strains were identified according to standard methods (Senior and Leslie, 1986) and stored on nutrient-agar slopes at 4°C. P/S typing and O serotyping of strains were performed as previously described (Larsson and Olling, 1977; Senior, 1977; Senior and Larsson, 1983).

Media

Nutrient Agar (Oxoid CM 3) and blood agar (BA) composed of Columbia Agar Base (Oxoid CM 331) with sterile horse blood (Oxoid SR 50) 5% v/v were prepared and sterilised according to the manufacturer's instructions. Cooked blood agar (CBA) was heated BA. Modified New York City Medium (MNYC) was prepared according to the method of Young (1978) but with horse blood (Oxoid SR 50).

Preparation of purified ¹²⁵I-labelled IgA

IgA was purified from frozen normal human plasma. After thawing, calcium chloride was added to a concentration of 20 mM and fibrin clots were removed by

Table. Characteristics of the *P. mirabilis* strains examined

Strain no.	P/S type	Source and clinical symptoms	O type	Origin
1 64676	3/1, 8, 13	urine—chronic urinary tract infection	O 28	Dundee
2 50213A	3/1, 8	urine—chronic urinary tract infection	O 3	Dundee
3 50253	3/1, 13	urine—chronic urinary tract infection	O 10	Dundee
4 SW 46	1/3	faeces—not known	O 24	Göteborg, Sweden
5 F40010	0/7	faeces—diarrhoea	O 41	Dundee
6 F59242	1/0	faeces—diarrhoea	O 27	Dundee
7 49704/80	2/1	ureter—ureter stone	...	Dundee
8 75844/77	5/0	kidney—renal stone	...	Dundee
9 33025N	3/10	cerebellum—abscess	...	London
10 PR 22	4/11	ear—chronic suppurative otitis media	...	Glasgow
11 OPF 10	3/4, 10	faeces—control healthy person	...	Dundee
12 EW 5	6/2, 3	sputum—not known	...	Bristol
13 U6450	3/1, 13	urine—chronic urinary tract infection	...	Wurzburg, Germany
14 81662/85	3/8, 13	vagina—pelvic sepsis	...	Dundee
15 PB 12	5/7	urine—chronic urinary tract infection	O 6	Portsmouth

filtration through glass wool. Ammonium sulphate was added to the resulting serum to 50% saturation. After standing overnight at 4°C, the precipitate was collected by centrifugation, dissolved in distilled water, applied to a G 200 Sephadex column and eluted with 50 mM Tris-HCl pH 8.0. IgA peak fractions were collected and purified by ion-exchange chromatography on a FPLC Mono Q column (Pharmacia) eluted with a linear gradient of 0.0–5 M NaCl in 50 mM Tris-HCl, pH 8.0. Peak IgA fractions were further purified by gel filtration on a FPLC Superose-6 column (Pharmacia) with phosphate-buffered saline pH 7.4. The resultant pure IgA was labelled with ¹²⁵I by the chloramine-T method of Hunter and Greenwood (1962).

Preparation of IgA protease

Circular disks (approximately 8.5 cm diameter) cut from dialysis tubing were sterilised in water at 121°C for 15 min and placed flat on to the surface of plates of culture media (BA for *P. mirabilis* strains, CBA for *H. influenzae* and *S. pneumoniae*, and MNYC for *N. gonorrhoeae*) by the use of a sterile cotton-wool swab. The surface of the membrane was inoculated with a cotton-wool swab containing one of the bacterial strains and the plates were incubated overnight at 37°C in the presence of CO₂ 8%. Bacteria were then scraped from the membrane with a microscope slide and transferred to a microcentrifuge tube. The membrane was rinsed with 0.5 ml of 5 mM Tris-HCl buffer pH 7.2 which was also added to the tube. The cells were emulsified in the buffer, centrifuged for 2 min at 11 600 *g* and the clear supernate containing IgA protease removed.

Digestion of ¹²⁵I IgA with IgA protease

¹²⁵I IgA (25 µg/ml of phosphate-buffered saline, pH 7.4) was incubated in 2.5-µl amounts with 50 µl of

IgA protease preparations in 5 mM Tris-HCl buffer, pH 7.2, for 20 h at 37°C. EDTA at a final concentration of 5 mM or phosphoramidon (Sigma, Dorset, England) at a final concentration of 2 µM was added to some reactions. After incubation, 25 µl of buffer (0.1 M Tris-HCl, pH 8.0, containing 80 mM dithiothreitol, 8 M urea, SDS 2% w/v and a trace of bromophenol blue dye) was added to each sample. After standing in boiling water for 2 min the samples were subjected to electrophoresis.

Polyacrylamide gel electrophoresis and autoradiography

Test samples and standard protein mol.-wt markers were analysed by SDS discontinuous polyacrylamide gel electrophoresis (PAGE) by the method of Laemmli (1970). The resolving gel was a linear gradient of 5–22.5% w/v acrylamide and electrophoresis was performed at 40 mA until the dye front reached the bottom of the gel. Gels were stained in Coomassie Brilliant Blue R250 0.125% w/v in methanol 50% v/v and glacial acetic acid 20% v/v in water for 1–2 h. They were subsequently destained in methanol 30% v/v and glacial acetic acid 10% v/v in water, dried under vacuum, and exposed to X Omat AR film at –70°C. After an appropriate period the film was developed.

Results

The PAGE analyses of the products of ¹²⁵I IgA digestion with enzymes produced by *P. mirabilis* strain 64676—a strain associated with chronic urinary tract infections—and those of *N. gonorrhoeae*, *H. influenzae* and *S. pneumoniae* are shown in fig. 1. The *P. mirabilis* enzyme cleaved the IgA heavy chain at positions different from the hinge region cleavage sites of the other bacterial IgA

Fig. 1. Polyacrylamide gel analysis of ^{125}I IgA digested with various microbial IgA proteases. The *P. mirabilis* protease is EDTA sensitive and cleaves the IgA heavy chain at sites different from those attacked by other bacterial proteases. Left; position of mol. wt standards from top to bottom; bovine albumin (66 000), ovalbumin (45 000), glyceraldehyde-3-phosphate dehydrogenase (36 000), carbonic anhydrase (29 000), trypsinogen (24 000), trypsin inhibitor (20 100), α lactalbumin (14 200). Tracks contain ^{125}I IgA cleaved with enzymes as follows: 1. control untreated IgA—H=heavy chain, L=light chain; 2. *P. mirabilis* strain 64676 protease; 3. *P. mirabilis* strain 64676 protease with 5 mM EDTA; 4. *P. mirabilis* strain 64676 protease with 2 μM phosphoramidon; 5. *N. gonorrhoeae* protease; 6. *N. gonorrhoeae* protease with 5 mM EDTA; 7. *H. influenzae* protease; 8. *H. influenzae* protease with 5 mM EDTA; 9. *S. pneumoniae* protease; 10. *S. pneumoniae* protease with 5 mM EDTA.

proteases. The mol. wts of the *P. mirabilis*-cleaved fragments were approximately 47 000 and 34 000. The *P. mirabilis* enzyme was inhibited by 5 mM EDTA but not by phosphoramidon, an inhibitor of several metalloproteinases (Umezawa and Aoyagi, 1977). The IgA protease of *S. pneumoniae* was also EDTA sensitive but the IgA proteases of *N. gonorrhoeae* and *H. influenzae* were not inhibited by 5 mM EDTA.

The kinetics of IgA degradation by the enzymes from *P. mirabilis* strain 64676 are shown in fig. 2. The IgA heavy chain (mol. wt 68 000) appeared to be cleaved to a fragment of mol. wt 47 000 in a single step, because larger fragments of intermediate size were not seen before the formation of the 47 000-mol. wt fragment. The kinetics of formation of the fragment of 34 000 mol. wt appeared to be synchronous with that of the fragment of mol. wt 47 000. This suggested that the smaller fragment

did not arise by secondary proteolysis of the larger fragment. Cleavage products of the IgA heavy chain smaller than mol. wt 34 000 were not observed.

Fifteen strains of *P. mirabilis* (see table), with 14 distinct P/S types and at least seven different O antigens, isolated from patients with various clinical symptoms and from healthy carriers in several geographical locations, all produced an IgA protease (fig. 3). The products of IgA cleavage by the enzyme from each strain were of the same size as those generated by the IgA protease from *P. mirabilis* strain 64676.

Discussion

IgA1 proteases are a group of enzymes that specifically cleave the heavy chain of the IgA1 subclass of human immunoglobulin A (IgA) at sites

Fig. 2. Polyacrylamide gel analysis of IgA degradation kinetics by *P. mirabilis* strain 64676 protease showing the simultaneous appearance of fragments of mol. wt 47 000 and 34 000. Lanes 1–7: ^{125}I IgA incubated with protease at 37°C. EDTA was added to samples of the mixture after (left to right) 0, 0.5, 1, 2, 4, 12 and 20 h to a final concentration of 5 mM to terminate the reaction; incubation was continued for a total of 20 h. Lane 8: ^{125}I IgA was incubated without protease for 20 h at 37°C (Control)—H = heavy chain; L = light chain.

within a 16-amino-acid proline-rich polypeptide in the hinge region. This polypeptide sequence is absent from IgA2 which, as a consequence, is resistant to the protease. IgA1 proteases are stably produced *in vitro* and *in vivo* (Sorensen and Kilian, 1984) by several human pathogens (Kilian *et al.*, 1983a), including *S. pneumoniae* (Kilian *et al.*, 1979; Male, 1979; Mulks *et al.*, 1980a), *N. gonorrhoeae* (Plaut *et al.*, 1975; Blake and Swanson, 1978), *N. meningitidis* (Plaut *et al.*, 1975; Mulks *et al.*, 1980b) and *H. influenzae* (Kilian *et al.*, 1979 and 1983b; Male, 1979; Mulks *et al.*, 1980a). Non-pathogenic members of the same genera do not produce IgA proteases (Mulks and Plaut, 1978; Kilian *et al.*, 1979; Male 1979) which suggests that this enzyme may be involved in virulence.

While investigating virulence mechanisms of *P. mirabilis* for the urinary tract, we found that the uropathogenic strain 64676 produced an enzyme able to cleave the IgA heavy chain to a fragment of mol. wt 47 000. Removal of carbohydrate from the

heavy chain by glycosidase activity would not result in such a reduction in mol. wt, whereas deglycosylation of the several carbohydrate chains would result in the formation of cleaved products intermediate in size between intact heavy chain and the observed final cleavage product. Such intermediates were not observed, which suggests that the enzyme was a protease rather than a glycosidase. There are also several possible explanations for the formation of two fragments from the heavy chain, but further studies are required before any other conclusions are made on the nature of this enzyme.

Since the size of the fragments cleaved by the enzymes from the other 14 strains of *P. mirabilis* of diverse type were indistinguishable from those cleaved by the *P. mirabilis* strain 64676 IgA protease, it appears that most strains of *P. mirabilis* produce a similar type of IgA protease. IgA protease production by *P. mirabilis* may, therefore, be an important virulence factor, not only in urinary tract infections but in infections at other sites.

Fig. 3. Polyacrylamide gel analysis of ^{125}I IgA digested with proteases from different *P. mirabilis* strains. The cleaved IgA fragments were similar in size to those resulting from cleavage with *P. mirabilis* strain 64676 protease. From the left are enzymic digests of IgA from strains in the same order as in the table. Control undigested IgA was in lane 8—H = heavy chain, L = light chain.

We confirmed previous findings (Kilian *et al.*, 1983a) that the IgA1 proteases of *N. gonorrhoeae* and *H. influenzae* are relatively resistant to EDTA whereas that of *S. pneumoniae* is EDTA sensitive. The *P. mirabilis* IgA protease was metal chelator-sensitive but differed from other IgA1 proteases by cleaving IgA outside the hinge region. It may,

therefore, also have activity on IgA2 which would be advantageous to a pathogen.

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