The production and activity *in vivo* of *Proteus mirabilis* IgA protease in infections of the urinary tract

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Summary. Immunoblotting of urine from 21 patients of both sexes and of wide age range who had a *Proteus mirabilis* urinary tract infection (UTI) showed that 14 (64%) specimens contained immunoglobulin A (IgA). In nine (64%) of these the IgA heavy chain had been degraded to fragments of a size identical to those formed when purified IgA was degraded by pure *P. mirabilis* protease. Urine from patients with clinical evidence of upper UTI contained fragmented IgA and in some of these urine samples *P. mirabilis* protease activity was detectable. Urine infected with a non-proteolytic strain contained only intact IgA. It is concluded that *P. mirabilis* IgA protease is produced and is active during infections of the urinary tract.

Introduction

Proteus mirabilis is a common cause of urinary tract infection (UTI) particularly in young boys^{1,2} and the elderly.³ The consequences of this may be serious because *P. mirabilis* has a predilection for the upper urinary tract.^{4,5} The virulence of *P. mirabilis* for the urinary tract arises through the interplay of several factors that include growth rate,⁶ motility,⁷ urease,^{8–12} possibly fimbriae,^{13,14} haemolysin^{15–18} and proticines or proticine receptors or both.³

Recently we reported that *P. mirabilis* strains of diverse type¹⁹ and some strains of other *Proteus* spp.²⁰ produce an edetic acid-sensitive protease that cleaves the heavy chain of immunoglobulin A (IgA) outside the hinge region. Subsequently we purified the protease and demonstrated its ability to degrade the heavy chains of both serum and secretory IgA₁, IgA₂ and IgG isotypes.²¹ Such a broad range of activity against these immunological body defences suggests that the protease may be another important virulence factor of *P. mirabilis*. Therefore, we investigated the production and activity *in vivo* of *P. mirabilis* protease in patients with UTI.

Materials and methods

Urine specimens

Specimens of midstream (MSU) or catheter (CSU) urine were selected at random from those sent for

routine bacteriological examination. Only specimens voided and delivered to the laboratory within 2 h, unless refrigerated, were examined. All these contained a pure growth of a *Proteus* spp. of $\geq 10^5$ cfu/ml for a MSU or an equal or lower count for a CSU. After isolation of the infecting organism, the urine specimens were stored at -20°C .

Isolation and identification of bacteria

The urine was plated on CLED Agar (Oxoid CM 301). After incubation overnight at 37°C , the infecting *Proteus* strain was isolated and identified as described previously.²²

Media

Casein-CLED agar consisted of CLED agar 36 g and casein (Sigma) 10 g in 1 L of 50 mM Tris. The casein was dissolved by vigorous stirring and the CLED agar was then added. Gelatin-CLED agar consisted of CLED agar 36 g and gelatin (Sigma, 300 bloom) 10 g in 1 L of 50 mM Tris. Both media were adjusted to pH 8.0, autoclaved at 121°C for 15 min and poured into plates.

Examination for proteolytic activity

Proteus isolates were inoculated on to the above media. After incubation for 16 h at 37°C , the plates were flooded with 1 N HCl. Caseinase and gelatinase activities were revealed as zones of clearing around the growth of proteolytic strains.

Preparation and purification of protease

P. mirabilis strain 64676 was grown overnight at 37°C on sterile dialysis-tubing membranes placed on Blood Agar (Columbia Agar Base, Oxoid CM 331, supplemented with horse blood 5%).¹⁹ The protease was purified from the washings of the cells and membranes by the method described previously.²¹

Purification of IgA₁

IgA₁ was purified from pooled normal human serum by affinity chromatography of IgA on Jacalin-Sepharose as previously described.²¹

Polyacrylamide gel electrophoresis of urine

Urine specimens were clarified by centrifugation at 11 600 *g* for 2 min. The supernate was removed and 50 µl was added to 50 µl of disruption buffer (100 mM Tris-HCl, pH 8.0, containing 8 M urea, sodium dodecyl sulphate, SDS, 2% and a trace of bromophenol blue dye) containing 80 mM dithiothreitol. The mixture was boiled for 2 min and 1 M iodoacetamide in disruption buffer was immediately added to the mixture to a final concentration of 100 mM. Protein mol.-wt markers and 60–100-µl volumes of the reduced urine samples were loaded on to a stacking gel of polyacrylamide 3% in 0.125 M Tris-HCl, pH 6.8, containing SDS 0.1% above a discontinuous separating gradient gel of acrylamide 5–15% in 0.375 M Tris-HCl, pH 8.9, containing SDS 0.2%. The upper tank buffer consisted of Tris 0.63%, glycine 0.39% and SDS 0.1%, pH 8.9, and the lower tank buffer consisted of Tris 1.2% and SDS 0.1%, pH 8.1. Electrophoresis was performed at 25 mA in the cold until the dye front reached the bottom of the gel.

Immunoblotting

The proteins separated on the polyacrylamide gel were transferred to a nitrocellulose membrane by electrophoresis in methanol 10% containing 25 mM Tris and 190 mM glycine at 4°C for 16 h at 10 V. The membrane was then stained with Ponceau S dye 0.2% in trichloroacetic acid 3% and the position of the mol.-wt proteins marked. After washing thoroughly in phosphate-buffered saline (PBS), the membrane was agitated for 2 h at room temperature in PBS containing dried milk powder (Marvel) 5% and then washed in several changes of PBS. It was then incubated with agitation in alkaline phosphatase-conjugated goat anti-human IgA (α -chain specific) (Sigma) diluted 1 in 200 in dried milk powder 5% in PBS containing azide 0.1% for 2 h at room temperature. The membrane was then washed for 30 min in several changes of PBS. The immunoblot was developed in 30 ml of buffer (100 mM NaCl, 5 mM MgCl₂ and 100 mM Tris-HCl, pH 9.5) containing bromochloroindolyl phosphate (Sigma) 5 mg and nitroblue tetrazolium (Sigma) 10 mg.

Detection of protease in urine

Urine specimens were clarified by centrifugation at 11 600 *g* for 2 min and 50 µl of the supernate was added to 50 µl of sample buffer (0.125 M Tris-HCl, pH 6.8, containing glycerol 20%, SDS 4%, mercaptoethanol 1% and a trace of bromophenol blue dye). The mixture was loaded on to a stacking gel of acrylamide 4% in 0.125 M Tris-HCl, pH 6.8, buffer containing SDS 0.1%, above a resolving gel of acrylamide 11% containing gelatin 0.1% in 0.375 M Tris-HCl, pH 8.9, buffer containing SDS 0.2%. The upper tank buffer was Tris 0.63%, glycine 0.39%, SDS 0.1%, pH 8.9. The lower tank buffer was Tris 1.2% and SDS 0.1%, pH 8.1. Electrophoresis was performed in the cold at 15 mA for 16 h.

To remove SDS, the resolving gel was washed twice in 500 ml of Triton X100 2.5% in water, each time for 1 h at 4°C, and then immersed in 50 mM Tris-HCl, pH 8.0, buffer and incubated for 4 h at 37°C. The gel was subsequently stained in Coomassie Brilliant Blue R 250 0.125% in methanol 50% and acetic acid 10% for 2 h and destained overnight in methanol 10% and acetic acid 10% in water. The presence and size of protease was indicated by the position of clear areas of unstained degraded gelatin against a blue background of undegraded stained gelatin.

Results

Twenty-two specimens of urine from 21 patients (10 male, 11 female) aged 4–97 years were examined (table). In each specimen the infecting organism was *P. mirabilis*. All the *P. mirabilis* isolates except strain 29139G were proteolytic and degraded both gelatin and casein.

The results of the immunoblots of 18 of the 22 urine specimens are presented in fig. 1. Purified protease from *P. mirabilis* strain 64676 degraded the α chain of purified IgA₁ (66 Kda) (lane 1) to two fragments of 60 and 47 Kda (lane 2). Eight (36%) urine specimens did not contain detectable IgA. Five (23%) urines contained different amounts of intact α chain and nine (41%) contained α chain that had been degraded to fragments of 60 and 47 Kda or a fragment of 47 Kda only. Specimen M, which was infected with the non-proteolytic strain of *P. mirabilis*, contained a significant amount of intact α chain but not chain fragments.

Electrophoretic analysis of urine on acrylamide-gelatin gels showed that four specimens contained active protease indistinguishable in size from that of pure *P. mirabilis* protease (fig. 2). These urine specimens were some of those in which α chain fragments were detected.

Discussion

Secretory IgA in mucous secretions protects mucous membranes from damage by micro-organisms and

Table. Specimens of *P. mirabilis*-infected urine examined for IgA and IgA fragments

Specimen	<i>P. mirabilis</i> isolate	Type of specimen	IgA		
			detected	intact	fragmented
A	02337R	CSU	+	+	
B	27831K	ND	+	+	
C	13102F	CSU	+		+
D	32262R	MSU	+	+	
E*	27729A	CSU	+		+
F	26071V	CSU	+		+
G	10966V	CSU	+		+
H	03060S	CSU	+		+
I	32404B	MSU	—		
J	17074G	CSU	+		+
K	15585X	MSU	—		
L	29878D	CSU	+		+
M	29139G	CSU	+	+	
N	29066K	CSU	+		+
O	17410Y	CSU	—		
P	28707N	MSU	—		
Q	32114W	ND	+		+
R	26927W	MSU	—		
S*	30691Y	CSU	+	+	
T	30298G	CSU	—		
U	28287K	CSU	—		
V	00030F	ND	—		

ND, urine specimen not defined.

* Specimens from same patient.

Fig. 1. Immunoblot of electrophoresed *P. mirabilis*-infected urine samples with alkaline phosphatase-conjugated goat anti-human α -chain-specific IgA. Lane 1, purified serum IgA₁; 2, purified serum IgA₁ digested with purified *P. mirabilis* strain 64676 protease to fragments of 60 and 47 Kda; A–R, *P. mirabilis*-infected urine specimens. Urine samples I, K, O, P, and R (and T, U and V not shown) did not contain detectable IgA. Urine samples A, B, D and M (and S not shown) contained different amounts of intact α -chain. Urine samples C, F, H, J, L and N contained α -chain fragments of 60 and 47 Kda and urine samples E, G and Q an α -chain fragment of 47 Kda.

Fig. 2. Demonstration of protease activity in infected urine specimens H (lane 1), J (2), L (4) and N (5) and purified protease from *P. mirabilis* strain 64676 (3). Samples were electrophoresed on polyacrylamide gels containing gelatin. Numbers on left refer to M_r (Kda) and the position of prestained mol.-wt protein markers.

their products by inhibiting adhesion and colonisation and by neutralising toxins and enzymes. Only a few bacterial pathogens produce enzymes capable of degrading IgA₁²³ and since other related but non-pathogenic species do not form IgA₁ proteases, their production may be associated with virulence.²⁴ The ability of these proteases to function *in vivo* is suggested by the demonstration of α -chain fragments in vaginal secretions from women with gonococcal infection and in cerebrospinal fluid from patients with *Haemophilus influenzae* meningitis.²⁵

The protease of *P. mirabilis* is somewhat different from other IgA₁ proteases because its activity is not

confined to the IgA₁ isotype but includes serum and secretory IgA₁, IgA₂, IgG and other non-immunoglobulin proteins including secretory component, casein and bovine serum albumin.²¹ Such a wider range of activity is clearly to the advantage of an organism seeking to establish infection.

We have, for the first time, shown here that 64% of urines from patients with *P. mirabilis* UTI in which IgA was detectable contained α -chain fragments of a size identical to those formed when the α -chain is degraded by pure *P. mirabilis* protease. Such fragments were not formed when the infecting strain was non-proteolytic. This is indirect evidence for the production and activity *in vivo* of *P. mirabilis* protease in UTI. The formation of α -chain fragments of 60 and 47 Kda is a feature of low protease activity or early degradation, whereas formation of a single fragment of 47 Kda is a feature of high protease activity or more extensive degradation (unpublished results).

Some of the urine specimens in which α -chain fragments were found were also shown by electrophoresis on gelatin-acrylamide gels to contain a protease of the same size as pure *P. mirabilis* protease. This is direct evidence of production *in vivo* of *P. mirabilis* protease in urine. Electrophoresis on acrylamide-gelatin gels is an extremely sensitive method of detecting protease. However, in spite of this, protease activity was not detected in some urine samples in which IgA fragments were found. We believe this to be the result of loss of enzyme activity on storage. The urine samples were stored at -20°C to preserve antibody and antibody fragments. Subsequently we have found protease activity to be more stable at 4°C .

The pH optimum for *P. mirabilis* protease is pH 8 (unpublished results). Therefore, under normal circumstances of infection, the infecting *P. mirabilis* strain will form urease, degrade urea and produce alkaline conditions that will permit the protease to act *in vivo* at its optimal rate. However, five patients had urine in which the IgA remained intact. For specimen M, this appeared to be because the infecting strain was non-proteolytic. For specimens A, B, D and S with pH values of 9, 8, 9 and 9 respectively, lack of α -chain degradation was not due to unfavourable pH. Failure to detect fragments in these specimens may have been because of insufficient protease to cause degradation, or too short an incubation period for degradation to have taken place. Alternatively, because it is thought that IgA protease activity may be subject *in vivo* to anti-protease antibody,²⁶ the IgA may have remained intact because antibody to the protease had been formed during a previous UTI with *P. mirabilis*. The latter hypothesis would explain why α -chain fragments were not detected in urine specimen S, though they had been present during an infection 17 days earlier (specimen E).

All specimens in which degraded IgA fragments were found came from CSU specimens from elderly patients (mean age 72 years) of both sexes with clinical evidence of UTI, although the bacterial count of some

was only 10^3 – 10^4 cfu/ml. On the other hand, urines in which IgA was not detected, were from younger patients (mean age 43 years) for whom clinical evidence of infection, before microbiological confirmation, was generally less clear, and they included four of the five MSU specimens. The significance of

these clinical observations must be established to understand more fully the in-vivo role of IgA protease in UTI.

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