BACTERIAL PATHOGENICITY

An investigation into the influences of species and biotype on the type of IgA1 protease produced by isolates of *Haemophilus*

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A total of 59 isolates of different *Haemophilus* spp., mostly from clinical specimens, was characterised, bityped and examined for production of type 1 or type 2 IgA1 protease. IgA1 protease activity was not found in any isolate of a species with no or low virulence for man including *H. parainfluenzae*, *H. haemolyticus*, *H. aphrophilus*, *H. paraphrophilus*, *H. segnis*, *H. paraphrohaemolyticus* and *H. haemoglobinophilus*. IgA1 protease was produced by all isolates of *H. influenzae* and *H. aegyptius* and by some isolates of *H. parahaemolyticus*. The type of IgA1 protease appeared to be independent of the biotype of the isolate in *H. influenzae*. For the first time some isolates of *H. aegyptius* were found that produced type 2 IgA1 protease. IgA1 protease production in *H. parahaemolyticus* may be associated with the virulence of the isolate.

Introduction

The mucosal surfaces of the body are protected from damage by micro-organisms and their products principally by the specific immunity provided by immunoglobulin A (IgA) in its secretory form (sIgA). sIgA neutralises toxins, enzymes and viruses, agglutinates bacteria and prevents bacterial adhesion to mucous membranes [1–4]. The ability of sIgA to perform its defensive effector functions depends upon its structural integrity. The physicochemical nature of sIgA renders it resistant to most types of proteolytic attack [5]. However, a few pathogenic bacteria that cause infections at mucous membranes are able to destroy its effector functions through the production of enzymes called IgA1 proteases [6–8]. They are so named because they cleave the α chain of IgA1 only and not that of the IgA2 isotype. These enzymes may be important virulence factors because they are produced *in vivo* [9, 10], patients convalescing from infections with IgA1 protease-producing bacteria have neutralising antibodies to the enzymes [11–13], and related but non-pathogenic bacteria do not produce them [7]. Most strains of *Haemophilus influenzae* produce serine-type IgA1 proteases that cleave the α chain in the hinge region at either Pro²³¹-Ser²³² (type 1 enzyme) or Pro²³⁵-Thr²³⁶ (type 2 enzyme) [14, 15]. The purpose of

this study was to examine IgA1 protease production among isolates of a wide range of *Haemophilus* spp. and determine whether or not there was any correlation between the type of enzyme formed and the species and biotype of the strain.

Materials and methods

Haemophilus strains and their isolation

Most (52 of 59) of the *Haemophilus* isolates studied were from clinical specimens. The majority (22, 42%) were from sputum, seven (13%) were from throat swabs and five (10%) were from eye swabs. The remainder (18, 35%) were from nasal and perinasal swabs, ear swabs, endotracheal aspirates and bronchial washings. The specimens were plated out for single colonies on cooked blood agar (CBA) plates and incubated overnight at 37°C in air with CO₂ 5%. Half of a well-isolated colony was picked and plated out on Columbia Agar (Oxoid CM 331). The other half of the colony was plated out on CBA. After incubation overnight at 37°C in air with CO₂ 5%, those strains that failed to grow on Columbia agar but grew on CBA and which were subsequently shown to be gram-negative short bacilli were saved as probable *Haemophilus* spp. Haemolytic species of *Haemophilus* were isolated differently. Blood agar (Blood Agar Base 'Oxoid CM 55' supplemented with horse blood 5%) plates (BA) were inoculated with swabs from patients with sore throats and incubated anaerobically overnight

at 37°C. Well-isolated haemolytic colonies were picked, re-plated on BA and V-factor growth disks were applied. After incubation overnight at 37°C in air with CO₂ 5%, isolates that gave haemolytic colonies whose size was increased by V factor and were gram-negative short bacilli were saved.

Single isolates of *H. haemolyticus*, *H. parahaemolyticus*, *H. aphrophilus*, *H. paraphrophilus*, *H. segnis*, *H. paraphrohaemolyticus* and *H. haemoglobinophilus* were purchased from the Haemophilus Reference Laboratory, Oxford Public Health Laboratory. The *Haemophilus* strains for daily use were maintained as pure cultures on CBA plates at room temperature and subcultured every 3–4 days. Permanent stocks were maintained in Tryptone Soya Broth (Oxoid CM 129) containing glycerol 15% at –70°C.

Media

Sugar fermentation test medium. Columbia agar (Oxoid CM 331) 3.9 g and phenol red dye 25 mg were added to 100 ml of distilled water and sterilised by autoclaving at 121°C for 15 min. While still molten but cool, the medium was supplemented with both the appropriate sugar (1%) from a filtered sterile 10% stock solution and also nicotinamide-adenine dinucleotide (NAD) and haemin (both 10 µg/ml) were added from sterile stock solutions of each (10 mg/ml). The complete medium was dispensed aseptically into the wells of a microtitration plate.

Ornithine decarboxylase medium. Bactopeptone (Difco) 0.5 g, yeast extract (Oxoid L21) 0.3 g, glucose, 0.1 g and phenol-red dye 25 mg were dissolved in 100 ml of distilled water and divided into two portions of 50 ml. L-Ornithine 0.5 g was added to one of these; the other served as the base control. Both were autoclaved at 121°C for 15 min and dispensed aseptically in small volumes into small sterile test tubes.

Indole test medium. This was tryptophan 0.1% in 50 mM sodium phosphate buffer, pH 8, sterilised by autoclaving at 121°C for 15 min.

Urease test medium. Phenol red dye (0.5 ml of 2%) was added to 100 ml distilled water in which K₂HPO₄ 0.1 g, KH₂PO₄ 0.1 g and NaCl 0.5 g had been dissolved and the pH of the solution was adjusted to 7. After autoclaving at 121°C for 15 min and when cool, the medium was supplemented aseptically with urea (2%) from a filtered sterile stock urea solution (40% in water).

Speciation and biotyping of strains

X and V growth factor requirement test. A suspension of the bacterial strain in saline was prepared with a swab from growth on a CBA plate and the same swab was used to streak the suspension across a Columbia

agar plate. X and V growth factor disks (Mast Diagnostics, Merseyside) were placed over the inoculum and c. 2.5 cm apart. After incubation overnight at 37°C in air with CO₂ 5%, the growth factor requirement of the strain was deduced from the areas in which it had grown.

ALA test. This test determined the ability of a strain to synthesise porphyrins from δ-aminolaevulinic acid (ALA). Strains were inoculated on to ALA discs (Mast Diagnostics) on a Columbia agar plate. After incubation overnight at 37°C in air with CO₂ 5%, the plate was examined under UV light. A brick-red fluorescence of the ALA disk indicated porphyrin synthesis from ALA.

Biotyping. All isolates were examined for their ability to form urease, indole and ornithine decarboxylase. The urea, indole and ornithine decarboxylase and base test media were inoculated with two drops of a dense suspension in saline of each strain from a CBA plate. The ornithine decarboxylase and base test media were then overlaid with sterile mineral oil. The reactions were read after incubation for 4 h at 37°C and the addition of Kovac's reagent to the indole test medium. Control strains giving appropriate positive and negative reactions were included in each batch of tests.

Preparation of IgA protease

Sterile dialysis tubing membrane on the surface of a CBA plate was inoculated by swab with an isolate. After incubation for 72 h at 37°C in air with CO₂ 5%, the membrane was removed and the growth from it was suspended in 0.5 ml of PBS buffer, pH 7.2, containing sodium azide 0.1%. The membrane surface was then rinsed in a further 0.5 ml of the buffer and the rinse was combined with the cell suspension. This was centrifuged at 12 600 *g* for 2 min. The supernate which contained any protease activity was removed and stored at –20°C.

Preparation of IgA1

CHO-K1 cells were grown by standard methods. These were Chinese hamster ovary cells bearing mouse genes for the immunoglobulin λ chain to the hapten NIP (3-nitro-4-hydroxy-5-iodophenylacetate) which had been transfected with the plasmid pJW6 which bears a mouse gene for the variable region of α chain to NIP and human genes for the CH1, CH2 and CH3 regions of human α1 chain [16]. The culture supernate was incubated overnight at 4°C with NIP-coated Sepharose beads. The beads were collected by filtration and, after thorough washing, recombinant IgA1 was eluted from the beads by competition with 1 mM NIP. The IgA1 was dialysed thoroughly against PBS containing sodium azide 0.1% and stored at –20°C.

Characterisation of IgA1 protease activity

Appropriate small volumes of recombinant IgA1 and IgA protease preparations were added to PBS, pH 7.2, containing sodium azide 0.1% to give a total volume of 15 μ l and incubated for 72 h at 37°C in a thermal cycler with a heated lid. After the addition of 15 μ l of 40 mM iodoacetamide in disruption buffer (8 M urea, SDS 2%, glycerol 20% and a trace of bromophenol blue dye in 125 mM Tris-HCl buffer, pH 6.8) and boiling for 5 min, the samples were loaded on to a stacking gel of acrylamide 4.5% in 125 mM Tris-HCl, pH 6.8, containing SDS 0.1% over a separating gel of acrylamide 10% in 375 mM Tris-HCl, pH 8.8, containing SDS 0.1% and electrophoresed at 150 V in electrophoresis buffer (25 mM Tris, 192 mM glycine, SDS 0.1% pH 8.3) until the dye front reached the bottom of the gel. The proteins were then transferred to a nitrocellulose membrane by electrophoresis for 1 h at 100 V in transfer buffer (25 mM Tris, 192 mM glycine in methanol 20% in water). The membrane was then blocked by agitation for 30 min in non-fat dried milk protein (Marvel) 5% in PBS. After thorough washing in PBS, the membrane was immersed in horseradish peroxidase-labelled sheep antibody to human α chain diluted 1 in 1000 in PBS containing Tween 20 0.1% and agitated for 2 h at room temperature. After thorough washing in PBS, the immunoblot was devel-

oped in 10 ml of 50 mM Tris-HCl, pH 7.6, containing nickel chloride 3 mg, diaminobenzidine 10 mg and hydrogen peroxide 30% 60 μ l.

SDS-PAGE of *Haemophilus* spp.

Several colonies from isolates grown on CBA were suspended in 1 ml of saline. After centrifugation at 11 600 *g* for 2 min, the washed cell pellet was resuspended in 100 μ l of distilled water and 100 μ l of sample buffer (glycerol 20%, SDS 4%, mercaptoethanol 10% and a trace of bromophenol blue dye in 0.125 M Tris-HCl buffer, pH 6.8). After being boiled for 5 min, portions of the preparations were electrophoresed on a polyacrylamide (10%) gel and stained as described previously [17].

Results

The clinical isolates were identified to species according to the criteria in Table 1 and biotyped on the basis of formation of indole, urease and ornithine decarboxylase according to the schemes of Kilian and others [18–21] (Table 2). At this stage, three strains that did not acidify xylose but were otherwise indistinguishable by biotyping from *H. influenzae* strains of biotype 3, were called *H. aegyptius*. These species were differ-

Table 1. Biochemical characteristics of the clinical isolates of *Haemophilus* spp.

Species	Growth factor requirement	ALA test*	Haemolysis†	Xylose‡	Sucrose‡
<i>H. influenzae</i>	X and V	–	–	+	–
<i>H. parainfluenzae</i>	V	+	–	–	+
<i>H. aegyptius</i>	X and V	–	–	+	–
<i>H. haemolyticus</i>	X and V	–	+	–	–
<i>H. parahaemolyticus</i>	V	+	+	–	+

*Synthesis of porphyrins from δ -aminolaevulinic acid.

†Haemolysis on horse blood agar plates.

‡Acidification of sugar.

Table 2. The types of IgA1 protease produced by different species and biotypes of *Haemophilus*

Species	Formation of			Biotype	Number of isolates	Number (%) producing IgA1 protease of	
	Indole	Urease	ODC			type 1	type 2
<i>H. influenzae</i>	+	+	+	1	7	2 (28.5)	5 (71.5)
	+	+	–	2	21	11 (52)*	9 (43)*
	–	+	–	3	7	2 (28.6)	5 (71.4)
	–	+	+	4	2	2 (100)	0 (0)
	+	–	–	7	1	1 (100)	0 (0)
<i>H. parainfluenzae</i>	–	+	+	2	2	0 (0)	0 (0)
	–	+	–	3	2	0 (0)	0 (0)
	+	+	+	4	1	0 (0)	0 (0)
	+	+	–	7	2	0 (0)	0 (0)
<i>H. aegyptius</i>	–	+	–	–	3	1 (33)	2 (66)
<i>H. haemolyticus</i>	–	+	–	–	3	0 (0)	0 (0)
<i>H. parahaemolyticus</i>	V	+	V	–	3	0 (0)	1 (33)
<i>H. aphrophilus</i>	NT	NT	NT	–	1	0 (0)	0 (0)
<i>H. paraphrophilus</i>	NT	NT	NT	–	1	0 (0)	0 (0)
<i>H. segnis</i>	NT	NT	NT	–	1	0 (0)	0 (0)
<i>H. paraphrohaemolyticus</i>	NT	NT	NT	–	1	0 (0)	0 (0)
<i>H. haemoglobinophilus</i>	NT	NT	NT	–	1	0 (0)	0 (0)

ODC, ornithine decarboxylase; NT, not tested; V, different isolates gave different results.

*One isolate produced both type 1 and type 2 enzyme.

entiated by SDS-PAGE of the major proteins of the three xylose-negative strains with seven *H. influenzae* biotype 3 strains as controls. The profiles of the *H. influenzae* strains were all very similar and had as major bands proteins of 39, 37.5 and 36.5 kDa (Fig. 1). However, those of the three xylose-negative strains were distinct from these and from one another. The most obvious differences, among others, in the xylose-negative strains were that strain 3 had major proteins of 39, 36.5 and 35.5 kDa, strain 5 had major proteins of 39, 38.5 and 34.5 kDa and strain 38 had major proteins of 37 and 35.5 kDa. It was concluded that the three xylose-negative strains were indeed strains of *H. aegyptius*.

As shown in Table 2, *H. influenzae* was the species most frequently isolated and comprised 38 (73%) of the 52 clinical isolates studied. *H. influenzae* biotype 2 strains were the most commonly encountered biotype (21 isolates, 55% of the total *H. influenzae* isolates), followed by biotypes 1 and 3 (seven isolates each, 18.4%). Strains of biotypes 4 and 7 were encountered less frequently. There was no obvious correlation between the biotype of the strain and the clinical specimen from which it was isolated.

Other species of *Haemophilus* were isolated less commonly. Among these were *H. parainfluenzae*

(seven isolates), *H. aegyptius* (three), *H. haemolyticus* (two) and *H. parahaemolyticus* (two).

All the clinical isolates, together with the strains from the reference laboratory (a total of 59 isolates of 10 species of *Haemophilus*), were examined for IgA1 protease production. IgA1 protease activity was detected on immunoblots of non-reduced samples as a protein band of either 53 kDa or 51.5 kDa representing the action, respectively, of a type 2 or a type 1 IgA1 protease (Fig. 2). The bands were the Fab fragments formed by cleavage of the α chain at either Pro²³¹-Ser²³² (type 1 enzyme) or Pro²³⁵-Thr²³⁶ (type 2 enzyme). The results of IgA1 protease production and the type of enzyme formed are presented in Table 2. From this it can be seen that IgA protease production was characteristic of all isolates of *H. influenzae*, regardless of their biotype, and was found in all *H. aegyptius* and one of three strains of *H. parahaemolyticus*. IgA protease production was not detected in any of the other *Haemophilus* spp. associated with man nor in *H. haemoglobinophilus*, whose natural host is the dog. Although all of the few isolates of *H. influenzae* of biotypes 4 and 7 produced only type 1 IgA protease, among the more numerous isolates of *H. influenzae* of biotypes 1, 2 and 3, isolates were found that formed either type 1 or type 2 IgA protease. In one case an isolate of *H. influenzae* of

Fig. 1. SDS-PAGE of the major proteins of *H. influenzae* biotype 3 isolates: Lane 1, mol. wt protein standards; 2, isolate 20; 3, 22; 4, 24; 8, 26; 9, 28; 10, 43 and xylose-negative *H. aegyptius* isolates; 5, isolate 3, 6, 5; 7, 38.

Fig. 2. Immunoblot of recombinant IgA1 after incubation with preparations from *Haemophilus* spp. and electrophoresis under non-reducing conditions showing Fab fragments produced by type 1 and type 2 IgA1 protease. Preparations and enzyme type were lane 1, *H. influenzae* biotype 1, isolate 27, type 1; 2, IgA1 control, no enzyme; 3, *H. influenzae* biotype 1, isolate 4, type 2; 4, *H. parainfluenzae* biotype 2, isolate 7; 5, *H. aphrophilus*; 6, *H. paraphrophilus*; 7, *H. influenzae* biotype 2, isolate 9, type 2; 8, *H. influenzae* biotype 2, isolate 6, type 1; 9, *H. haemolyticus*; 10, *H. influenzae* biotype 3, isolate 20, type 1; 11, *H. influenzae* biotype 4, isolate 41, type 1; 12, *H. aegyptius* isolate 3, type 1; 13, *H. aegyptius* isolate 5, type 2; 14, *H. influenzae* biotype 3, isolate 22, type 2; 15, *H. parahaemolyticus* isolate 50, type 2; 16, *H. influenzae* biotype 7, isolate 25, type 1; 17, *H. influenzae* biotype 2, isolate 31, type 1; 18, *H. influenzae* biotype 2, isolate 33, type 2; 19, *H. segnis*.

biotype 2 formed both enzymes simultaneously. When the biotype of the isolate was disregarded, almost exactly the same number of *H. influenzae* isolates formed type 1 protease as formed the type 2 enzyme. Thus, it appeared that, in *H. influenzae*, the biotype of the strain probably had no influence on the type of IgA1 protease formed.

One of the three *H. aegyptius* isolates produced a type 1 IgA1 protease and two produced a type 2 enzyme (Fig. 2). This is believed to be the first recorded detection of type 2 IgA1 protease production by *H. aegyptius*. One of the three isolates of *H. parahaemolyticus* produced IgA1 protease of type 2.

Discussion

H. influenzae is a major pathogen of man and virtually all isolates – regardless of the presence or absence of capsules, capsule serotype, biotype and origin – have been shown to produce IgA1 protease [22–25]. Indeed, even in the few isolates that do not produce the enzyme, the *iga* gene sequence can be detected in the genome [26]. The results presented here, which showed that all the isolates of *H. influenzae* from a variety of sources whose capsulate state and type were unknown produced IgA1 protease, substantiate this fact. Most strains form a type 1 or a type 2 enzyme, but occasionally a strain is found that produces both types of protease simultaneously [14]. Although non-capsulate strains of *H. influenzae* produce IgA1 protease, there is a strong correlation between the capsular serotype of the strain and the type of enzyme formed. Thus most strains of capsular serotypes A, B and D form type 1 enzyme, those of serotypes C and E form type 2 enzyme [25]. However, although the *H. influenzae* isolates in the present study were not

capsule serotyped, the biotyping results suggest that, by way of contrast, there is little or no correlation between *H. influenzae* biotype and the type of enzyme formed. This is in agreement with the findings of Mulks *et al.* [25]. Both type 1 and type 2 proteases were found to be produced by *H. influenzae* isolates of biotypes 2 and 3 and, although all the *H. influenzae* isolates of biotypes 4 and 7 produced only type 1 protease, there were few isolates of these biotypes; examination of more isolates may have detected some that produced type 2 enzyme. This kind of problem may be the reason why others [25] found all of three strains of *H. influenzae* of biotype 1 to produce type 1 enzyme, whereas among the seven biotype 1 isolates in the present study, two produced type 1 enzyme and five produced type 2. When the biotype of the *H. influenzae* isolate was disregarded, the numbers of isolates producing type 1 and type 2 enzymes were almost the same.

H. aegyptius is also a known pathogen of man and is associated with conjunctivitis and Brazilian purpuric fever [27]. *H. aegyptius* strains share >90% DNA sequence homology with *H. influenzae* [27] and have so many properties in common with *H. influenzae* that many now call them *H. influenzae* biogroup *aegyptius*. Despite the close similarity between *H. influenzae* biotype 3 strains and *H. aegyptius* strains, the two can be distinguished. The best discriminating test is analysis of their outer-membrane proteins [28]. The SDS-PAGE results in the present study supported the view that the xylose-negative isolates were different from the *H. influenzae* biotype 3 strains and were indeed isolates of *H. aegyptius*. Because of these similarities with *H. influenzae*, it was not surprising to find that all the *H. aegyptius* isolates produced IgA protease. This finding is in agreement with the work of others [14, 29]. However, all the *H. aegyptius*

strains so far reported [14] produce a type 1 protease, for they cleave at the same site (Pro²³¹-Ser²³²) on the α chain as that of type 1 protease of *H. influenzae* strains. However, in the present study two of the three isolates of *H. aegyptius* produced a type 2 protease. Although this is the first report of type 2 IgA1 protease production by *H. aegyptius* it is not an unexpected finding, because the closely related *H. influenzae* biotype 3 isolates produced either a type 1 or a type 2 protease.

IgA protease production was not detected in isolates of *H. parainfluenzae*, *H. haemolyticus*, *H. aphrophilus*, *H. paraphrophilus*, *H. segnis*, *H. paraphrohaemolyticus* or *H. haemoglobinophilus* despite incubation of culture supernates with recombinant IgA1 for 72 h. Although for some of these species only single isolates were examined, the results support the findings of others [22–24, 29]. These species are considered to have low or no pathogenicity for man and this finding gives support to the hypothesis that IgA protease production is a virulence factor for pathogenic species of *Haemophilus*.

IgA protease production was also detected in one of three isolates of *H. parahaemolyticus*. Others have also reported that a minority of strains of *H. parahaemolyticus* form IgA protease [23, 24, 29]. This organism is commonly found in the oral cavity and pharynx of most healthy people [30] and as such is often regarded as non-pathogenic. However, it is also one of the commonest isolates from purulent infections of the oral cavity [31]. This may indicate that some isolates of this organism may be pathogenic. Kilian [18] has shown that *H. parahaemolyticus* isolates can be separated into two groups on the basis of biochemical reactions. It would be interesting to determine if IgA protease production is restricted to the isolates from purulent infections and whether or not these have other biochemical activities that distinguish them from the commensal isolates.

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