BACTERIAL PATHOGENICITY

Lack of interference between IgA-binding proteins and IgA proteases of human pathogenic bacteria

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Some human bacterial pathogens produce specific immunoglobulin A1 (IgA1) proteases that cleave the heavy chain of human IgA1, generating intact Fab and Fc fragments. Other pathogenic bacterial species express surface proteins which bind to the Fc part of human IgA in a non-immune manner. To analyse whether IgA-binding proteins affect the activity of IgA1 proteases, the ability of seven different IgA1 proteases to hydrolyse IgA1 in the presence of either of two different bacterial IgA-binding proteins was tested. Data obtained in two different types of experiment suggest that IgA1 bound to IgA-binding proteins still functions as a substrate for IgA1 proteases. As Fc fragments produced by cleaving IgA1 with IgA1 proteases still bind to IgA-binding proteins, we conclude that these two types of bacterial protein act independently of each other.

Introduction

Since the first description of an IgA protease produced by *Streptococcus sanguis*, several clinically important human pathogens that colonise and infect human mucosal surfaces have been reported to produce specific IgA1 proteases [1]. The three leading causative agents of bacterial meningitis (*Neisseria meningitidis*, *S. pneumoniae* and *Haemophilus influenzae*) all produce IgA1 proteases, whereas closely related, non-pathogenic species do not [2, 3]. Other clinically important bacteria known to produce IgA1 proteases include *N. gonorrhoeae*, some oral streptococci and some *Bacteroides* and *Ureaplasma* spp. [1, 2]. The IgA1 proteases expressed by these bacteria share the ability to hydrolyse one of the peptide bonds in a duplicated octapeptide present in the hinge region of the $\alpha 1$ heavy chain. As the $\alpha 2$ chain lacks this amino-acid sequence, it is resistant to hydrolysis by IgA1 proteases. Except for the autocatalytic activity exhibited by some IgA1 proteases, IgA1 from man and a few other primates is the only known substrate for IgA1 proteases [1]. However, the IgA1 proteases constitute a structurally diverse group of molecules. The nucleotide sequences of the IgA1 protease genes (*iga*) of *N. meningitidis*, *N. gonorrhoeae* and *H. influenzae* are homologous [4] and

code for large, 106–108-kDa, proteins [5, 6], but these proteins show considerable differences with regard to molecular and serological properties [7]. The reported sequence for the *iga* gene of *S. sanguis* codes for a 190-kDa protein and shows no similarity to *iga* genes from gram-negative bacteria [8]. Furthermore, the gene fails to hybridise the chromosomal DNA from *S. pneumoniae* [9].

Another way for human pathogens to interact with IgA is to express surface proteins that bind to the Fc part of IgA in a non-immune manner. Such proteins are expressed by many strains of *S. pyogenes* and by some strains of *S. agalactiae* [10]. Two of the IgA-binding proteins expressed by *S. pyogenes* have been extensively characterised with regard to immunochemical properties—protein Sir that binds both IgA and IgG, and protein Arp that preferentially binds IgA [10, 11]. The overall structure of these IgA-binding proteins is similar; they have molecular masses of 37–45 kDa [11–13] and they are members of the M protein family [14]. In contrast, the IgA-binding 124-kDa protein expressed by *S. agalactiae* (designated protein Bac or the β antigen) is immunologically and structurally unrelated to the IgA-binding proteins of *S. pyogenes* [10, 15]. Although protein Bac has been shown to bind almost exclusively to serum IgA and not to secretory IgA [15], this IgA-binding protein was included in the present study as protein Bac is structurally unrelated to the IgA-binding proteins of *S. pyogenes*.

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Bacterial species expressing IgA-binding proteins do not produce IgA proteases [2], and no IgA1 protease-positive bacteria are known to produce IgA-binding proteins of Arp or Bac type. However, bacteria that express IgA proteases and bacteria that express IgA-binding proteins are commonly found simultaneously in the upper respiratory tract [16]. Therefore, it was of interest to analyse whether these large bacterial proteins interact, as they are produced by major human pathogens. Previously reported results had shown that the bacterial IgA-binding proteins bind to purified Fc-fragments produced by digestion with an IgA protease [10, 17], but this finding did not exclude the possibility that the IgA-binding proteins might interfere with the action of IgA proteases. Furthermore, it was of interest to analyse the purified IgA-binding proteins for protease activity.

Materials and methods

IgA1 proteases and IgA-binding proteins

Seven different IgA1 proteases were tested: the enzymes from *S. sanguis*, *S. pneumoniae*, the type 1 and type 2 enzymes from *H. influenzae* and *N. meningitidis* and the type 2 enzyme from *N. gonorrhoeae*. The proteases were purified as described previously [18]. Protein Sir, the IgA- and IgG-binding protein expressed by a group A streptococcal strain of serotype M22, was purified after expression in *Escherichia coli* as described previously [11]. Protein Bac was purified from a group B streptococcal type Ib strain as described previously [15]. Protein G (Calbiochem-Novabiochem, San Diego, CA, USA), an IgG-binding protein originating from the group G streptococcus, was used as a control. The IgA used as substrate in this study was the monoclonal IgA1 (Mor) protein [19], which was ^{125}I -labelled by the chloramine-T method [20]. Proteins Sir and Bac are known to bind a majority of human monoclonal IgA proteins (ref. 11 and our unpublished data). To confirm that these proteins bind to IgA1(Mor), proteins Sir and Bac were separated by SDS-PAGE, transferred to a polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore Corp., Bedford, MA, USA) and incubated with ^{125}I -IgA1 (Mor). Both bacterial proteins reacted strongly with the probe, whereas the IgG-binding protein G lacked reactivity in a similar way (data not shown).

IgA protease assays

The soluble phase assays were performed at room temperature with mixtures of ^{125}I -IgA, antibody binding protein, and diluted enzyme in 20 mM Tris-buffer, pH 7.2, supplemented with 10 μM MgCl_2 and 10 μM CaCl_2 in the assays with proteases from gram-positive bacteria. To allow binding of IgA to IgA-binding protein, 15 μl of ^{125}I -IgA (0.5 μg) was pre-incubated with 15 μl of IgA-binding protein (0.6 μg of Sir or 1.5 μg of Bac, i.e., four-to-five-fold molar excess of

IgA-binding protein), control protein (protein G, 0.6 μg) or buffer for 1 h at room temperature. After this pre-incubation, 15 μl of enzyme in solution was added, and samples of the reaction mixture were removed at different time points. The reactions were stopped by the addition of sample buffer (SDS 2% and 2-mercaptoethanol 5%) and the samples were boiled for 5 min. The assay was performed with two incubation times and with at least two different enzyme concentrations for each enzyme. The samples were separated by SDS-PAGE in acrylamide 12% gels and the gel was dried and subjected to autoradiography and phosphor imaging (Molecular Dynamics, Sunnyvale, CA, USA) to allow quantification of radioactivity.

In the second type of assay, immobilised pre-formed complexes of IgA-binding protein and ^{125}I -IgA were digested with different IgA1 proteases. Proteins Sir (8 μg), Bac (4 μg), G (8 μg) or ^{125}I -IgA (0.4 μg) were immobilised on a PVDF membrane, and the membranes were blocked and washed with PBS supplemented with Tween 20 0.25% and gelatin 0.25%, and incubated with ^{125}I -IgA for 16 h at 8°C. After incubation, the membranes were washed four times for 20 min each with PBS supplemented with Tween 0.25%, gelatin 0.25%, 1 M NaCl and 10 mM EDTA. Eight identical membranes were prepared and each membrane was incubated for 5 h at room temperature with one of the seven enzymes tested, or with buffer alone. After incubation, the membranes were washed and dried, and the radioactivity was quantified by phosphor imaging.

Results

The potential interference of IgA-binding proteins with the activity of IgA1 proteases was tested in a standard assay. In different experiments the incubation times and the amount of enzyme used was varied, allowing complete or only partial cleavage of the α chain. The enzymic activity of the protease was not affected by the presence of IgA-binding protein in any of these experiments. Fig. 1 shows the result of a representative experiment in which the effect of the IgA-binding proteins Sir and Bac was analysed, with the IgG-binding protein G as control. Inspection of the autoradiographs revealed no differences in the amount of cleaved fragment produced in the absence or presence of IgA-binding proteins. The amount of radioactivity in the bands representing the uncleaved and the cleaved α chain was quantified by phosphor imaging, and the ratio of the two values (cleaved:uncleaved) was used as an estimate of protease activity. The results showed that the cleavage reaction was equally efficient in the presence of protein Sir, Bac or G as when buffer alone was added. Only the reaction with *N. gonorrhoeae* enzyme type 2 in low concentration showed a slight inhibitory effect (25% lower ratio than in control; data not shown). Similar results were

Fig. 1. Effect of bacterial IgA-binding proteins on the activity of IgA1 proteases. Mixtures of ^{125}I -IgA, IgA1 protease and IgA-binding protein were incubated for 1 h at room temperature before separation by SDS-PAGE. After separation, the gel was dried and subjected to autoradiography. The reactions analysed on this gel were designed to allow only partial digestion of the $\alpha 1$ chain. Letters indicate the enzyme used: A, *S. sanguis* IgA1 protease; B, *S. pneumoniae* IgA1 protease; C, *H. influenzae* IgA1 protease type 1. The IgA-binding protein (or control) used is indicated above each lane. The lane marked ^{125}I -IgA contains uncleaved ^{125}I -IgA. The positions of uncleaved $\alpha 1$ chain (upper arrow) and an NH_2 -terminal fragment of the $\alpha 1$ chain (lower arrow) are indicated.

obtained when the IgA-binding protein Arp [10] was used as potential inhibitor (data not shown). These results clearly indicate that pre-incubation of ^{125}I -IgA with an IgA-binding protein does not affect the ability of IgA proteases to cleave the IgA.

In the experiment shown in Fig. 1, IgA-binding proteins were present in a four-to-five-fold excess as compared to IgA-Fc. Since the binding of IgA to protein Sir and protein Bac is of high affinity [11, 15], it is unlikely that the ^{125}I -IgA digested was present in free, non-bound form. However, an experiment was also designed to analyse whether pre-formed complexes between IgA and IgA-binding proteins are sensitive to proteases. In this experiment, complexes formed on a membrane were used as the substrate (Fig. 2). Protein Sir and protein Bac were immobilised in a row of dots on a strip of PVDF membrane. After blocking and incubation with ^{125}I -IgA, the sensitivity to IgA1 protease of ^{125}I -IgA bound *via* protein Sir or protein Bac was analysed. Eight identical membrane strips were prepared and seven of these were incubated with different IgA proteases and one was incubated with buffer alone. The amount of enzyme added was expected to give 75–100% digestion of IgA in

solution. After incubation, the filters were washed and dried, and the amount of radioactivity remaining in the dots was quantified by phosphor-imaging radiography. The radioactivity in the control dots (membrane incubated with buffer alone) was compared with the radioactivity in the dots on the membranes digested with various enzymes. The complexes of IgA-binding protein and IgA were sensitive to cleavage, as the amount of radioactivity was reduced on all filters incubated with enzyme, as compared to the filter incubated with buffer alone. As c.10% of the radioactivity of the ^{125}I -IgA1(Mor) molecule is located on the Fc part [19], cleavage of all IgA molecules would result, theoretically, in a 90% reduction of radioactivity. However, radioactivity was not reduced to this extent, which may be explained by reduced activity of the IgA1 proteases close to the PVDF membrane. In an attempt to estimate the activity of the IgA1 protease under the experimental conditions used in Fig. 2, the activity of the protease on ^{125}I -IgA immobilised directly to the membrane was also determined (Fig. 2, black bars). The *H. influenzae* type 1 enzyme, which was the least efficient in cleaving IgA that had bound to immobilised IgA-binding proteins, was also the least efficient in cleaving IgA immobilised directly

Fig. 2. Effect of IgA1 proteases on pre-formed complexes between IgA1 and IgA-binding proteins. ^{125}I -IgA was immobilised on PVDF membrane strips *via* membrane-bound protein Sir (□) or protein Bac (▨). A series of identical strips was incubated with different IgA1 proteases or with buffer alone. The vertical bars show the amount of radioactivity retained after incubation with these enzymes. The amount of radioactivity on the membrane treated with buffer alone was defined as 100%. The activity of the different IgA1 proteases on ^{125}I -IgA immobilised directly to the PVDF-membrane was also determined (■) to estimate the activity of the different proteases under these experimental conditions.

to the membrane, suggesting that the PVDF membrane had an inhibitory effect on the protease activity. Nevertheless, the data in Fig. 2 show that a large fraction of the ^{125}I -IgA bound to IgA-binding proteins was sensitive to the action of the different IgA1 proteases, which indicated that the IgA-binding proteins did not interfere with the activity of IgA1 proteases.

To analyse whether the IgA-binding proteins, Sir, Arp or Bac had any IgA protease activity, these proteins were analysed in the IgA protease assay, with incubation times from 30 min to 4 h, and incubation temperatures of 37°C or 23°C. Samples were separated on SDS-PAGE and analysed by phosphor imaging as described above. In all cases, the ^{125}I -IgA used as substrate was not degraded by the presence of the IgA-binding proteins (data not shown). Furthermore, proteins Sir and Bac were shown to be resistant to the *S. sanguis* and *H. influenzae* type 2 IgA1 proteases (not shown).

Discussion

The production of IgA in man exceeds that of all other Ig classes combined [21]. Most of this IgA is found in secretions on mucosal surfaces, where it plays an important role in defence against pathogenic micro-organisms by virtue of its antigen-binding capacity. However, many pathogens interact with IgA by secreting highly specific IgA1 proteases [1, 3], and other bacteria express surface proteins capable of binding to the Fc part of IgA [10]. The biological role of these large bacterial proteins is not fully understood, but they are generally believed to be important for microbial virulence. The object of this study was to determine whether bacterial IgA1 proteases and IgA-binding proteins act independently on the IgA molecule.

Two different types of experiment were used, in which IgA substrate was complexed with IgA-binding proteins in solution, or immobilised on membranes. When ^{125}I -IgA was pre-incubated with either IgA-

binding protein or buffer prior to digestion with different IgA1 proteases, the enzymes were found to be equally active in the presence or absence of IgA-binding proteins, as determined by analysis of the cleaved fragments on SDS-PAGE. In the other type of experiment, complexes of IgA-binding proteins and ^{125}I -IgA were immobilised on a membrane. Incubation with various IgA1 proteases reduced the amount of radioactivity on the membrane, indicating that the complexed ^{125}I -IgA still functioned as a substrate for IgA1 protease. Taken together, these results indicate that IgA1 proteases and IgA-binding proteins, despite their large sizes, do not sterically interfere with each other; specifically, IgA-binding proteins fail to block access of proteases to the susceptible IgA1 hinge region peptide bonds. Furthermore, these results show that purified IgA-binding proteins lack IgA protease activity. Therefore, human bacterial pathogens colonising mucosal surfaces have developed two unrelated ways to interact with IgA, which underlines the importance of IgA in the defence against pathogens on mucosal surfaces.

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