The cleavage of immunoglobulin G \textit{in vitro} and \textit{in vivo} by a proteinase secreted by the urinary tract pathogen \textit{Proteus mirabilis}

L. M. LOOMES, M. A. KERR and B. W. SENIOR*

Departments of Pathology and *Medical Microbiology, Dundee University Medical School, Ninewells Hospital, Dundee DD1 9SY

Summary. Eighteen different strains of \textit{Proteus mirabilis} were all shown to produce an EDTA-sensitive proteinase of c. 50 kDa that cleaved the heavy chain, but not the light chain, of IgG. Digestion of pure IgG with small amounts of pure \textit{P. mirabilis} proteinase generated Fab\textsubscript{c}, and Fab\textsubscript{a} fragments; greater amounts generated Fab and Fc fragments that were comparable in size to those generated by pepsin and papain, respectively. Incubation of neutrophils with IgG digested with \textit{P. mirabilis} proteinase or papain resulted in a marked decrease in the respiratory burst activity of the neutrophils that coincided with cleavage of the IgG into Fab and Fc fragments. Analysis of urine from patients with \textit{P. mirabilis} urinary tract infection revealed in many the presence of Fab and Fc fragments of IgG indistinguishable in size from those generated by \textit{P. mirabilis} proteinase. These results indicate that, in \textit{P. mirabilis} urinary tract infections, the proteinase is secreted and cleaves IgG to fragments that have defective immune effector functions, thereby limiting the effectiveness of the immune response.

Introduction

Many micro-organisms pathogenic to man are now known to have strategies for avoiding the immune system of the host. \textit{Staphylococcus aureus} and some \textit{Streptococcus} spp. for example, express proteins on their surface that are able to bind immunoglobulins by the Fc region, thereby eliminating their Fc-mediated effector functions.\textsuperscript{1} Protein A and protein G that bind IgG have been extensively characterised\textsuperscript{2} and others that bind IgA have also been studied.\textsuperscript{3}

Some pathogens secrete proteinases that degrade immunoglobulins. Some of those that invade mucous membranes, e.g., \textit{Neisseria gonorrhoeae}, \textit{N. meningitidis}, \textit{Haemophilus influenzae}, \textit{Str. pneumoniae} and \textit{Ureaplasma urealyticum}, produce highly specific proteinases that degrade only IgA1. These enzymes are thought to be directly involved in pathogenicity because IgA1 fragments produced by the enzymes have been detected in body fluids of patients infected with these organisms,\textsuperscript{4,5} whereas organisms of related species considered to be non-pathogenic for man do not produce them.\textsuperscript{6}

Some other micro-organisms secrete proteinases of a broader specificity that are able to cleave both IgG and IgA \textit{in vitro}.\textsuperscript{7} We have shown that \textit{Proteus mirabilis}, a common urinary tract pathogen, secretes a proteinase that cleaves IgA1, IgA2 and IgG but not IgM.\textsuperscript{10} The enzyme has been purified by affinity chromatography on phenyl sepharose and characterised as a unique metalloproteinase.\textsuperscript{11} Moreover, active enzyme and IgA fragments produced by the enzyme can be detected in the urine of infected patients.\textsuperscript{12}

In this study, the action of the proteinase of diverse \textit{P. mirabilis} strains on IgG both \textit{in vitro} and \textit{in vivo} in patients with urinary tract infection was investigated. The composition and biological activity of the fragments produced was also studied, in an attempt to understand better how the proteinase may act as a virulence factor.

Materials and methods

Bacterial strains

The 18 \textit{P. mirabilis} strains examined were selected from a large collection of clinical isolates so as to provide a group with diverse characteristics. It comprised strains from Sweden, Germany and four British cities isolated from specimens of urine, faeces, sputum and pus from an ear. The strains belonged to at least 11 different O serotypes and 16 proticine production/sensitivity (p/s) types\textsuperscript{13} (table). The strains were speciated by standard methods\textsuperscript{14} and stored on nutrient-agar slopes in screw-capped bottles at 4°C.

Examination of strains for proteolytic activity

Each \textit{P. mirabilis} strain was incubated overnight at
Table. Characteristics of the *P. mirabilis* strains studied

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Origin</th>
<th>Source</th>
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ND, not determined; NT, not typable.

*Proticine production (p)/ sensitivity (s) type.

37°C in nutrient broth (Oxoid; CM 67), then a loopful of culture was inoculated on to buffered gelatin-CLED agar (Oxoid; CM 301) prepared as described previously. After incubation at 37°C for 16 h, the plates were flooded with 1 n HCl. Proteolytic strains were those whose growth was surrounded by a clear zone of degraded gelatin.

**Production of proteinase**

Proteinase was prepared from each *P. mirabilis* strain by culture both on solid and in liquid media. In the former method, strains were cultured overnight at 37°C on sterile dialysis tubing membranes placed on Blood Agar (Columbia Agar Base, Oxoid CM 331, supplemented with horse blood 5%). The bacteria were scraped from the membrane with a microscope slide and suspended in 50 mM Tris-HCl buffer, pH 8.0, containing sodium azide 0.1%. The membranes were washed thoroughly in the buffer and the washings added to the suspension. After centrifugation, the clear supernate containing protease was collected. In the latter method, strains were cultured with aerotrope in nutrient broth for 24 h at 37°C. After centrifugation, the supernate containing protease was collected and supplemented with sodium azide to a concentration of 0.1%.

**Azocaseinase assay of protease activity**

Buffer (50 mM Tris-HCl, pH 8.0) was added to a known volume (up to 25 μl) of the sample to give a final volume of 75 μl. After the addition of 50 μl azocasein 5 mg/ml in water, the mixture was incubated in a water bath at 37°C, usually for 1 h. The reaction was terminated by the addition of 250 μl of trichloroacetic acid (TCA) 5% w/v in water. After standing for a few minutes, the unhydrolysed precipitated azocasein was removed by centrifugation at 11600 g for 2 min. All the clear supernate was collected and added to a cuvette holding 375 μl of 0.5 M NaOH. After mixing, the absorbance at 440 nm was determined against a water blank. One unit of proteinase was defined as the activity hydrolysing 1 mg of azocasein in 1 h and was calculated from the equation: units of proteinase = A440 × 60/1.6 × incubation time (min).

**Analysis of proteinase**

The size characteristics of the proteinase of each strain were determined by the electrophoresis of 0.01 units of the proteinase on polyacrylamide-gelatin gels according to the method described previously.

**Purification of *P. mirabilis* proteinase**

The proteinase of *P. mirabilis* strain 64676 was purified from the filtered supernates of nutrient-broth cultures incubated at 37°C for 24 h by phenyl-Sepharose affinity chromatography as described previously.

**Purification of IgG**

IgG was purified from fresh, pooled, normal human serum as described previously. In essence, the serum proteins were dissolved in distilled water, after precipitation by 50% saturated ammonium sulphate, applied to a Sepharose 6B gel filtration column and eluted with 20 mM Tris-HCl (pH 8.0). The fractions containing IgG, as detected by radial immunodiffusion, were pooled and applied to a column of DEAE-Sepharose equilibrated in this buffer. Pure IgG was collected from the fractions eluted from the column with 20 mM Tris-HCl (pH 8.0). Portions of pure IgG were labelled with 125I by the chloramine T method of Greenwood et al.

**Cleavage of 125I-IgG with *P. mirabilis* proteinase**

Pure *P. mirabilis* 64676 proteinase (PMP; 50 μl) or 50 mM Tris-HCl buffer (pH 8.0; 50 μl) as a control, were incubated with 0.15 μg (10^4 cpm) of 125I-IgG for 64 h at 37°C. The preparations were then electrophoresed on polyacrylamide gels under reducing conditions and subsequently autoradiographed for 6 days.

**Cleavage of unlabelled IgG with proteolytic enzymes**

Portions (368 μg) of pure IgG were digested with: suitably diluted pure PMP in 0.1 M Tris-HCl buffer (pH 8.0); pepsin 0.125 mg/ml in 0.1 M sodium acetate buffer (pH 5.0); or papain 0.5 mg/ml in PBS containing 0.01 M cysteine and 2 mM EDTA, pH 7.1. The mixtures were incubated in a water bath at 37°C for various periods up to 72 h. At intervals, samples containing 15 μg of IgG were removed and the reactions were terminated by boiling for 5 min. The
IgG CLEAVAGE BY P. MIRABILIS

Fig. 1. Proteinase activity detected in SDS polyacrylamide-gelatin gels after electrophoresis of culture supernates of 18 strains of *P. mirabilis*. The supernates were diluted with buffer so that each had the same activity against azocasein.

Fig. 2. Autoradiograph of SDS polyacrylamide gel after electrophoresis under reducing conditions of \(^{125}\text{I}\)IgG incubated for 64 h at 37°C in either cultures of 18 different *P. mirabilis* strains (lanes 1–18) or buffer (lane 19—control C). The mobilities of IgG heavy chain (H), light chain (L) and the 31-kDa fragment of the heavy chain are indicated.

Samples were electrophoresed subsequently on polyacrylamide 5–20% gels under reducing and non-reducing conditions.

**Polyacrylamide gel electrophoresis (PAGE)**

SDS-PAGE was performed as described by Laemmli\(^{19}\) with resolving gels of a gradient of acrylamide of either 5–15% or 5–20% and a stacking gel of acrylamide 3%. Samples for electrophoresis that needed reducing were boiled for 2 min with an equal volume of 100 mM Tris-HCl (pH 8.0) containing 8 M urea, SDS 2%, 80 mM dithiothreitol and a trace of bromophenol blue dye; immediately afterwards, iodoacetamide was added to a final concentration of 80 mM. Unreduced samples were boiled for 2 min with an equal volume of 100 mM Tris-HCl (pH 8.0) containing 8 M urea, SDS 2%, 40 mM iodoacetamide and a trace of bromophenol blue dye. Electrophoresis was at 30 mA until the dye front reached the bottom of the gel. Gels for autoradiography were dried under vacuum on to filter paper and exposed at \(-40\)°C to Cronex 7 (Du Pont Co.) film in a cassette with double intensifying screens.

**Immunoblotting**

The proteins separated on polyacrylamide gels were transferred to a nitrocellulose membrane by electrophoresis in methanol 10% containing 25 mM Tris and
Fig. 3. Immunoblot of SDS polyacrylamide gel after electrophoresis under non-reducing conditions of IgG incubated for 24 h at 37°C with either buffer alone (left lane in each block) or with *P. mirabilis* strain 64676 proteinase. The blots were either (A) stained with Ponceau S to detect all proteins and peptides or incubated with alkaline phosphatase antibody to (B) γ chain or (C) κ and λ light chain and developed. Reactions contained 2 µg of enzyme and 5-160 µg of IgG. Equal amounts (5 pg) of IgG from the reactions were applied to each lane. Lane M shows the mobility of mol. wt markers of 91.4, 66.2 and 21.5 kDa.

Measurement of respiratory burst of neutrophils treated with IgG and IgG fragments

Samples (258 µg) of pure IgG were incubated with either 6 µg of pepsin or 6 µg of papain and 64.5 µg of pure IgG was incubated with 9 µg PMP under the conditions detailed above and at 37°C. At intervals over 72 h, samples (20 µl) containing 40 pg of IgG in the case of the reactions with pepsin and papain, and 10 µg of IgG in the case of the reaction with PMP, were removed, and the reactions were terminated by the addition of 2 M Tris to 0.25 M in the case of the pepsin reaction, 1 M iodoacetamide to 0-125 M in the papain reaction and 0.1 M EDTA to 25 mM in the PMP reaction.

PBS (200 µl; 8.5 mM sodium phosphate, 0-15 mM NaCl, pH 7.1) was added to each sample and 20 µl of the mixture was saved for subsequent analysis by SDS-PAGE. One hundred µl of the remainder was added in duplicate to microtiteration luminometer plates (Dynatech, Billingshurst, Sussex). After the plates had been incubated overnight at 4°C and washed three times in PBS, 100 µl of luminol (Sigma) (0.01 mM in Hanks's Balanced Salts Solution [Gibco] containing bovine serum albumin 1 mg/ml) HBSS-
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Fig. 5. The effect of incubation of IgG with (▲) papain, (■) pepsin and (●) P. mirabilis strain 64676 proteinase on its ability to interact with neutrophil Fc receptors and create a respiratory burst of chemiluminescence. After incubation of IgG with enzyme, the digests were coated on to microtitration luminometer wells and their ability to induce a chemiluminescent burst from human neutrophils determined. Values are the mean of duplicate wells expressing maximal chemiluminescence response as a percentage of control incubations (0 h) which contained IgG and no enzyme.

BSA) and human neutrophils (50 μl of 10^6 cells/ml in HBSS-BSA), purified from heparinised blood of healthy volunteers by centrifugation on Ficoll-Hypaque as described by Albrechtsen et al. were added to each well. Chemiluminescence was determined in a Dynatech luminometer ML 1000 as described every 5 min until the burst of respiratory activity had diminished to background level.

Analysis of urine

The urine specimens examined were selected from those sent for routine bacteriological examination of urinary tract infection. They had been refrigerated within 2 h of voiding and contained a pure growth of P. mirabilis at ≥ 10^6 cfu/ml for a midstream (MSU) or an equal or lower count for a catheter (CSU) specimen of urine. The urine was clarified by centrifugation at 11 600 g for 2 min. The supernate was removed and proteins precipitated from it by the addition of TCA to give 10% w/v. The protein precipitate was collected by centrifugation at 11 600 g for 2 min and dissolved in disruption buffer (100 mM Tris-HCl, pH 8.0, containing 8 M urea and SDS 2%) usually to 0.1 of the urine volume and stored at -20°C. Before electrophoresis, the sample was mixed with an equal volume of 100 mM Tris-HCl, pH 8.0, containing 80 mM dithiothreitol, glycerol 20% and a trace of bromophenol blue dye. The mixture was boiled for 2 min and then supplemented immediately with iodoacetamide in disruption buffer to 80 mM.

Results

Proteinases secreted by P. mirabilis strains

All the 18 strains (table) showed the typical biochemical reactions of P. mirabilis and proteolytic activity on gelatin-CLED plates. For each strain, the active protease found in the supernate of nutrient-broth cultures after incubation for 24 h at 37°C was detected as a protein doublet of 53- and 50-kDa after electrophoresis and development on polyacrylamide-gelatin gels (fig. 1).

When the supernates were incubated with ^125^I-IgG and the mixtures were subsequently electrophoresed on polyacrylamide gels under reducing conditions, the autoradiographs showed (fig. 2) that, for each strain, some of the IgG heavy chains were cleaved to give a fragment of c. 31 kDa lying just above the light chain. In every instance the light chain was not cleaved. The supernates of each strain also cleaved the heavy chain of ^125^I-IgA (results not shown). The rate of cleavage of IgA by the proteinase of each strain was always greater than the rate of cleavage of IgG. The proteolytic
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activity of each strain was inhibited by 10 mM EDTA. There was a clear correlation between the ability to cleave IgG and IgA and the azocaseinase activity of the supernates. Thus, the proteinases of all the diverse types of _P. mirabilis_ were indistinguishable with respect to size, activity and susceptibility to EDTA.

The proteinase of _P. mirabilis_ strain 64676 was chosen at random for further study and was purified by affinity chromatography on phenyl-sepharose.

**Specificity of IgG cleavage**

The specificity of IgG cleavage was studied in more detail by digesting pure unlabelled IgG with pure _P. mirabilis_ 64676 proteinase (PMP). The fragments produced were identified after separation by SDS-PAGE by immunoblotting with anti-heavy (y) and anti-light (\(\alpha\) and \(\kappa\)) specific antisera. The results (fig. 3) show that IgG digested with PMP gave, under non-reducing electrophoresis conditions, fragments of 130 kDa and 110 kDa corresponding to Fab'\(\gamma\) and Fab'\(\alpha\), and smaller fragments of 50 kDa and 43 kDa were also produced. The 50-kDa fragment contained only heavy chain determinants; the 43-kDa fragment contained only light chain determinants. This suggests that they correspond to Fc and Fab fragments, respectively, and that the anti-heavy chain antiserum recognised predominantly determinants in the CH2 and CH3 domains.

The amounts of the 130 kDa- and 110 kDa-fragments produced did not seem to vary with the concentration of PMP suggesting that these fragments were not further degraded into the smaller fragments. The amounts of the 50- and 43-kDa fragments produced increased as the substrate concentration (fig. 3) and enzyme concentration (not shown) increased. In contrast to our findings when radiolabelled IgG was digested and a low-mol.-wt fragment (14 kDa) was formed, when unlabelled IgG was digested, a 14-kDa fragment could not be detected either by immunoblotting or by staining the blots with gold stain. This suggests that iodination protected this small fragment from further degradation.

Comparison with the fragments generated by cleavage of IgG with pepsin and with papain confirmed (fig. 4) that PMP generated some Fab'\(\gamma\) fragments, but in the presence of more PMP, the major fragments were Fab and Fc.

**Effect of cleavage of IgG on biological activity**

The loss of effector functional activity of IgG resulting from cleavage by PMP, pepsin or papain was studied by measuring the ability of the fragments produced to induce a burst of chemiluminescence when incubated with purified human neutrophils. Fig. 5 shows that intact IgG, when aggregated by binding to the plastic luminometer plates, produced a respiratory burst of luminescence from the neutrophils. This ability was greatly diminished when neutrophils were incubated with IgG which had been digested with pepsin. Analysis of the incubation mixtures by SDS-PAGE showed that the loss of activity corresponded in time with the cleavage of IgG to produce Fab'\(\gamma\) fragments. Incubation of neutrophils with IgG that had been digested with papain or PMP resulted in a marked decrease in respiratory burst activity to a level approximately one-half of that stimulated by intact IgG. Analysis of the incubation mixtures by SDS-PAGE showed that, in each case, the IgG had been cleaved to Fab and Fc fragments.

This indicated clearly that the Fc fragment produced by PMP was functionally equivalent to that produced by papain. The new lower level of chemiluminescent activity corresponded to the activity of the Fc fragment bound to the microtitation plate and aggregated by the binding. Purified Fc, but not Fab, fragments from papain digestion, when bound to microtitation plates, elicited a similar response (results not shown).
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Fig. 7. The structure of IgG1 showing the sites of cleavage for papain, pepsin and P. mirabilis strain 64676 proteinase. Pepsin also degrades part of the Fc portion to small peptides leaving a dimer of the C_{H3} domain called pFc'.

**Analysis of infected urine for in-vivo cleavage of IgG**

When *P. mirabilis*-infected urine from each of 62 patients of both sexes and an age range of 4–83 years was examined, serum proteins including IgG and IgA, as determined by immunoblotting, were detected in many specimens. In contrast to IgA, the amount of IgG present was usually directly related to the amount of proteinuria. All the *P. mirabilis* isolates but one were found to be proteolytic. Intact or cleaved \( \gamma \) chain was detected in 26 (42%) of the specimens. A typical result of the analysis of the urine of five male and three female patients is presented in fig. 6. The cleavage of IgG was into Fab and Fc fragments of a size identical to those produced by PMP. The IgG was not degraded in the specimen infected with the non-proteolytic isolate. In every specimen in which both IgA and IgG were present and the IgG was cleaved, IgA fragments were also detected. These results indicate that in infections of the urinary tract, the proteinase of *P. mirabilis* strains is both secreted and is active in vivo on IgG (and IgA) in urine.

**Discussion**

Our results show clearly that all 18 clinical isolates of *P. mirabilis* of diverse types produced a proteinase that was able to cleave IgG. The enzyme appeared to account for all of the azocaseinase activity of the bacteria and to be the same as that which cleaves IgA. However, IgA cleavage appeared to occur at a more rapid rate than that of IgG. The major cleavage site of IgA by the enzyme is between the CH2 and CH3 domains yielding Fabc' fragments. However, the major site of cleavage of IgG by the enzyme is at the hinge, resulting in the production of Fab and Fc fragments which appear to be identical to those generated by papain. In addition, earlier cleavage events by smaller amounts of the enzyme produced Fabc' and Fabc' fragments (fig. 7). With prolonged incubation, these fragments were not cleaved further, nor did they accumulate. It seems probable that these fragments were the result of cleavage of a minor subclass of IgG present in the preparation.

The detection of both IgA fragments, and, as reported here, IgG fragments, in *P. mirabilis*-infected urine indicated that the proteinase was secreted and was active in vivo. Therefore, it may act as a virulence factor permitting the by-passing of the immune effector functions of these immunoglobulins. Its cleavage of IgG will yield Fab fragments which, if they are of sufficient affinity, will still bind to the bacterial cell. However, these fragments will not be able to elicit effector functions mediated by the Fc part of the molecule. In addition, they may block the binding of intact IgG molecules and thereby further limit the effectiveness of the immune response. Although the Fc fragments could be aggregated artificially in vitro, and
mediate effector functions, there is no evidence that such aggregation can occur in vivo and therefore the \textit{P. mirabilis}-cleaved IgG can be assumed to be inactive.

Although the mechanisms of effector functions mediated by IgA are not as well understood, our evidence (unpublished observation) suggests that the Fabc'$_3$ fragment, produced by cleavage of IgA by \textit{P. mirabilis} proteinase, is unable to bind to Fc$\alpha$ receptors on human myeloid cells. The detection of fragments of IgC and IgA as well as active enzyme in urine containing plasma proteins also suggests that the \textit{P. mirabilis} proteinase is not efficiently inhibited by any of the many proteinase inhibitors in plasma.

We are grateful for the financial support of the Scottish Home and Health Department.

References

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