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Retained Antigen-Binding Activity of Fab_α Fragments of Human Monoclonal Immunoglobulin A1 (IgA1) Cleaved by IgA1 Protease

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Immunoglobulin A1 (IgA1) proteases may be important virulence factors of certain bacteria involved in the pathogenesis of meningitis, gonorrhea, destructive periodontal diseases, and some other infections affecting mucosal membranes. This study evaluated the antigen-binding activity of free Fab_α fragments released from human myeloma IgA1 by IgA1 protease from *Haemophilus influenzae*. Six myeloma proteins with antibody activity against streptolysin O, α-staphylolysin, or streptococcal hyaluronidase were used. Complete cleavage of the IgA1 myeloma proteins in the hinge region of the heavy chain did not affect their antigen-binding capacity. The titers of neutralizing activity associated with free Fab_α fragments were not significantly different from those of the intact IgA1 proteins. The retained antigen-binding capacity of cleaved IgA1 is an important factor in the understanding of how IgA1 proteases may interfere with the immune protection of mucosal membranes.

The antigen-combining sites of immunoglobulins are located in the Fab parts of the intact molecule. Fab and $(Fab')_2$ fragments of immunoglobulin G (IgG) obtained by enzymatic digestion with papain and pepsin, respectively, retain the ability to bind the corresponding antigen, whereas the secondary reactivities mediated by the Fc region are lost. Other proteolytic enzymes have been shown to split immunoglobulins similarly, leaving Fab or $(Fab')_2$ fragments with unchanged antigen-binding properties. As an exception, Plaut et al. (20) found that monomeric Fab_α fragments released from five human IgA1 monoclonal antibodies after exposure to IgA1 proteases of *Neisseria gonorrhoeae* and *Streptococcus sanguis* had negligible antigen-binding activity. The five IgA1 paraproteins used by Plaut et al. (20) all had the characters of autoantibodies.

IgA1 proteases with the specific capacity to cleave the α1 heavy chain in the hinge region are excreted by several bacterial species that cause important infections in humans. There is considerable indirect evidence to suggest that IgA1 proteases play a role in the pathogenesis of bacterial meningitis, gonorrhea, destructive periodontitis, and certain other infections affecting mucosal membranes (for reviews, see references 13, 19). However, the exact mechanism by which cleavage of IgA1 into monomeric Fab and Fc fragments may promote the mucosal colonization and possible penetration by bacteria is not known. The ability of IgA1 proteases to affect the antigen-binding capacity of released Fab_α fragments is an important part of this problem.

Antibody activity against a bacterial antigen is occasionally found in human monoclonal proteins of the IgA class. This study describes the retained antigen-binding activity of Fab_α fragments obtained by IgA1 protease-induced cleavage of six monoclonal IgA1 proteins of the three following antibody specificities: anti-streptolysin O (SLO), anti-α-staphylolysin, and anti-streptococcal hyaluronidase (ASH).

MATERIALS AND METHODS

IgA M components. Six human IgA1 M components purified from patient sera by preparative block electrophoresis (see below) were used in this study. These IgA1 proteins, which had antibody activity against SLO, α-staphylolysin, or streptococcal hyaluronidase, were originally detected during the routine screening of patient sera for antibodies against these antigens. Some of the M components have previously been described (17, 18, 23). The antibody specificity and titers detected in the patient sera are summarized in Table 1. The titers of ASH, anti-SLO, and anti-α-staphylolysin were determined as described by Faber (5) and Ipsen (8, 9). The gammopathies detected in two patients, no. 3 and 5, were biclonal as these sera contained monoclonal IgG in addition to the IgA1 myeloma protein. The monoclonal IgG of these patient sera had the same antigen specificity and light chain types (17, 18).

Preparative block electrophoresis. Preparative block electrophoresis was carried out in agarose gel according to Jaton et al. (10). The LKB Multiphor electrophoresis equipment (LKB, Bromma, Sweden) was used with the tray for preparative isoelectric focusing (LKB 2117-501) as support for the agarose block (24.4 by 10.9 by 0.6 cm). The agarose was prepared as a 0.5% (wt/vol) solution of equal parts of LSA and LSB agarose (Litex, Copenhagen, Denmark) in Tris-Veronal buffer, pH 8.6 (ionic strength, 0.02) (24). A 3-ml portion of dialyzed serum was mixed with 3 ml of 1% melted agarose at 45°C and applied to a trough (10 by 100 mm) cut in the block. The electrophoretic separation was carried out for 24 h at 14°C and at a field strength of 3.5 to 4 V/cm. The separated proteins were recovered from 1-cm slices of the gel according to the described procedure (10).

Detection of antibody activity. ASH was determined by the turbidimetric method described by Faber (5). Combined hemolysis inhibition and immunoelectrophoresis (HII) was performed with preparations of α-staphylolysin and SLO as described previously (15, 16). The technique allows correlation of an immunoelectrophoretic analysis with detection of anti-hemolysin activity. In brief, cleaved or uncleaved preparations of the respective purified IgA proteins were subjected to electrophoresis in 1.5% agar, resulting in a clear separation of Fab_α and Fc_α fragments. A trough cut on one side of the sample well and filled with an appropriate antiserum (see below) allowed a traditional demonstration of

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TABLE 1. Characteristics of IgA1 myeloma proteins used

Patient	Antibody activity to:	Antibody titer (IU) per ml of serum	Reference
1	Streptolysin O	20,000	Unpublished
2	Streptolysin O	64,000	Unpublished
3	Streptolysin O	125,000	17
4	α-Staphylolysin	6,400	18
5	α-Staphylolysin	400	18
6	Streptococcal hyaluronidase	5,200,000 U	23

the position of IgA fragments (Fig. 1). On the opposite site, 2 mm from the sample well, a trough (4 by 65 mm) was cut and filled with 1.5% agar containing 5% rabbit erythrocytes (Fig. 1C to E). Finally, a 2-mm-wide trough 1 mm distal and parallel to the latter was filled with 0.1 ml of α-staphylolysin (0.3 U/ml) or 0.2 ml of SLO (7 U/ml). A progressive lysis of the erythrocytes occurred due to the gradual diffusion of α-staphylolysin except where its activity was neutralized by specific antibodies associated with the intact or cleaved IgA1 myeloma proteins. The inhibition zones were read after a diffusion time of 20 to 24 h. The SLO was applied in the oxidized inactive state. After diffusion overnight the SLO was reduced by immersing the plate in a 12.5 mM neutral cysteine solution. After 1 to 2 h of incubation the inhibition zones, in which the erythrocytes are protected by the neutralizing antibodies, were readily seen with a background of lysed cells.

Antisera. The antisera used for immunoelectrophoretic analyses and for the HII assay were anti-human serum, mono-specific antisera against alpha and gamma heavy chains and kappa and lambda light chains (Dakopatts, Copenhagen, Denmark), and an unabsorbed antiserum raised in rabbits against purified colostral secretory IgA (SIgA). An antiserum specific for Fc_α was prepared by absorption of an anti-alpha chain serum (Dakopatts) with an excess of Fab_α fragments purified from myeloma IgA1 as previously described (12).

Agarose gel electrophoresis. Agarose gel electrophoresis was carried out as described by Johansson (11).

IgA1 protease. A preparation of IgA1 protease from *Haemophilus influenzae* HK 393 (biotype I, serotype *b*) was prepared as described previously (12). Diluted 1:10 in substrate solution, the protease preparation was capable of causing complete cleavage of 1 mg of IgA1 myeloma protein within 1 h at 37°C as determined by immunoelectrophoretic analysis of the digestion products (Fig. 1A and B).

Samples of the respective purified myeloma proteins dissolved to a concentration of 2 mg/ml in 0.15 M phosphate buffer, pH 7.4, were incubated for 2 h at 37°C with an equal volume of *H. influenzae* IgA1 protease. Complete cleavage of the IgA1 protein induced by the enzyme was confirmed by immunoelectrophoretic analysis of the reaction mixture (Fig. 1A and B).

RESULTS

The six IgA myeloma proteins were purified to homogeneity as revealed by immunoelectrophoretic analysis. All six myeloma proteins were susceptible to *H. influenzae* IgA1 protease. This was demonstrated by a change in electrophoretic mobility of the monoclonal proteins in agarose gel as a result of enzyme treatment. Furthermore, immunoelectrophoretic analysis with chain-specific antiserum showed a change from one precipitate of IgA before digestion to two separate precipitates of Fab_α and Fc_α after digestion (Fig. 1A). The identity of Fab_α was confirmed by the use of appropriate light-chain-specific antisera (Fig. 1B).

Cleaved and uncleaved preparations of the IgA1 myeloma proteins that had anti-SLO (three samples) and anti-α-staphylolysin (two samples) activity were examined by the HII technique, using SLO and α-staphylolysin, respectively, as the hemolysin. The analyses showed that the Fab_α fragments retained their ability to inhibit the hemolysis of rabbit erythocytes by the hemolysin to which the myeloma protein had antibody activity (Fig. 1D). The association of the inhibitory activity with free Fab_α fragments was verified in two ways. (i) The inhibitory activity was detected in the gel at the position of free Fab fragments (Fig. 1D). (ii) In situ admixture of Fc_α-specific and light chain-specific antisera to cleaved IgA before electrophoresis had the following effects:

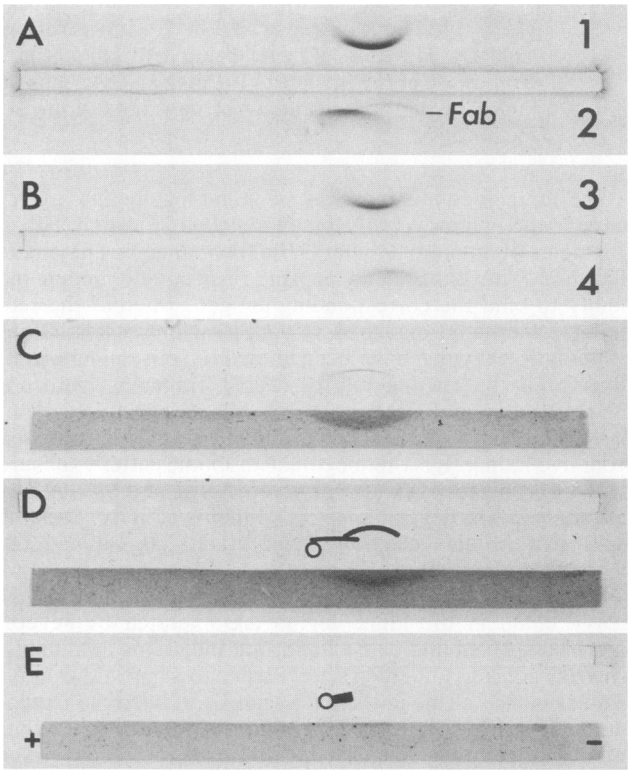

FIG. 1. Immunoelectrophoretic analysis of one of the purified IgA myeloma proteins with anti-SLO activity (wells 1 and 3) and of the same protein after IgA1 protease-induced cleavage into monomeric Fab_α and Fc_α fragments (wells 2 and 4). Antisera applied in the troughs were (A) anti-colostral SIgA and (B) anti-lambda light chains. (C), (D), and (E) illustrate analyses by the HII assay of the IgA1 protein and its cleavage products. Antiserum used in all troughs was anti-SIgA. The samples used in (C) and (D) were diluted to obtain suitably sized inhibition zones. As a result, the precipitates drawn on (D) and (E) were visible on the original plates, but too weak to be seen on the prints. The dark rectangles represent 1.5% agar gel containing 5% rabbit erythrocytes. The samples applied in the sample holes were: (C) purified IgA myeloma protein, (D) IgA1 protease-treated IgA, and (E) in situ admixture of anti-lambda light chains and the protease-treated IgA as in (D). At (C) and (D) hemolysis inhibition zones facing the intact IgA and the Fab_α, respectively, are seen. In (E) a partially soluble complex of Fab_α and anti-lambda antibodies is precipitated by the antiserum applied in the upper trough. The resulting antigen-antibody complex is unable to diffuse into the indicator zone of erythrocytes and, as a result, a hemolysis inhibition zone is not seen.

(a) Fc_α-specific antiserum, which caused precipitation of free Fc_α fragments, was unable to interfere with the inhibition zone; and (b) appropriate anti-light chain sera precipitated Fab_α fragments and made them practically unable to diffuse into the agar, with the result that no inhibition zone was observed (Fig. 1E).

An estimation of the titers from the size of the individual inhibition zones (18) observed in the HII assay with paired IgA and Fab_α indicated that no substantial loss of antibody activity had occurred as a result of IgA1 protease-induced cleavage.

A more exact quantitation of the effect of IgA1 protease-induced cleavage on antibody activity was performed with the myeloma protein that possessed anti-streptococcal hyaluronidase activity (patient 6). Preparations of the myeloma protein adjusted to contain approximately 10,000 U of anti-hyaluronidase activity (ASH) were incubated for 2 h at 37°C with 1 volume of either IgA1 protease or buffer. The antibody titers in the two preparations were subsequently determined by the turbidimetric assay described by Faber (5). A slight increase in ASH titer was observed after cleavage of the myeloma protein (titer, 7,000) versus the control (titer, 5,700).

DISCUSSION

All bacterial IgA1 proteases examined in detail cleave the IgA1 molecule at a specific peptide bond located in the hinge region of the heavy chain, leaving Fab and Fc fragments intact (13, 19). Being monovalent, the released Fab fragments cannot form cross-linked networks with antigen and are, therefore, incapable of inducing precipitation or agglutination. This is in keeping with the previous observation by Plaut and co-workers (20) that IgA1 paraproteins with anti-IgG activity (rheumatoid factor) lose the ability to agglutinate IgG-coated erythrocytes after cleavage with IgA1 protease. Plaut et al. (20), however, explained this by an essential loss of antigen-binding activity of released Fab_α fragments. The results presented here are at variance with that conclusion. Thus, our study showed that the enzyme-neutralizing activity of six IgA1 myeloma proteins was unchanged after cleavage with IgA1 protease and that this activity was associated with free Fab_α fragments.

Determined by the antibody specificity of the IgA1 proteins available for examination, the two studies used widely different assays to elucidate the effect of IgA1 protease-induced cleavage. In their study, Plaut et al. (20) found that neither radiolabeled Fab_α nor Fc_α fragments of IgA1 myeloma protein with anti-IgG activity bound significantly to IgG-coated erythrocytes, since isotope counts were only 10% of those using intact IgA. The concentration of IgA used in the assay was 1 mg/ml. However, this concentration of pure monoclonal antibody significantly exceeds the point of equivalence between antigen and antibody and is likely to result in a significant overestimation of intact IgA bound. This is particularly the case if the IgA was in a polymeric or aggregated form. Likewise, significant excess of antibody may also, in part, explain their observations that preincubation of IgG-coated erythrocytes with Fab_α fragments did not interfere detectably with a subsequent agglutination induced by intact IgA.

The myeloma proteins available for the present study provided the advantage that the antigen-binding capacity of intact IgA and free Fab_α fragments could be compared quantitatively in a sensitive biological system, i.e., enzyme or hemolysin neutralization. Our demonstration of a retained antigen-binding and neutralizing capacity of free Fab_α fragments is in agreement with classical studies of the biological functions of IgG fragments. The results, furthermore, corroborate the observations by Mallett and co-workers (14), who used preparations of purified SIgA which included antibodies to *S. mutans* antigens. Fab_α fragments prepared with *S. sanguis* IgA1 protease retained their binding capacity and deterred the sucrose-dependent adherence of *S. mutans* to glass. Additional support for this conclusion comes from some observations made in Plaut's laboratory. In attempts to prove the antibody nature of the IgA1 protease-inhibitory activity found in some samples of colostral SIgA, Gilbert et al. (7) showed that, after fragmentation of SIgA, the inhibitory activity was recovered in Fab_α-containing fractions.

We did not, in this study, examine the possibility that cleavage of IgA1 antibodies may affect their affinity. Cleavage of the molecule in the hinge region might cause a structural change in the released Fab_α fragments that would alter their affinity to the respective antigen. However, the results of our comparative determination of hyaluronidase-neutralizing activity associated with Fab_α fragments and intact IgA do not suggest a major change of affinity. In fact, a minor increase in hyaluronidase-neutralizing activity was observed in our experiments. This is probably due to an ability of each of the released Fab_α fragments to neutralize individual molecules of antigen.

It is therefore unlikely that bacterial IgA1 proteases interfere with antibody function of IgA1 by abolishing its antigen-binding capacity. Several other possibilities exist. First, the adherence-inhibitory activity ascribed to SIgA (6, 25) may well be dependent on the total SIgA molecule as suggested by recent studies on the mechanisms of antibody-induced neutralization of influenza virus (22). While SIgA antibodies are capable of reducing the attachment of avian influenza virus to cells grown in vitro and prevent it from being internalized, monomeric IgA antibodies and IgG lack this capacity (22). The same is likely to be the case for Fab_α fragments. Second, the mere loss of cross-linking ability of SIgA when cleaved by IgA1 protease is likely to have an effect on its protective properties. Thus, studies of experimental cholera in the infant mouse have demonstrated that reduction of $F(ab')_2$ fragments of IgG to Fab' is accompanied by a more than 10-fold decrease in protective activity. The loss of bi- (or tetra-) valency is also likely to result in a reduced binding strength of the interaction with antigen (3). Finally, important, but yet unknown, functions of the Fc_α part of the IgA1 molecule may be lost as a result of IgA1 protease-induced cleavage.

The demonstrated retained antigen-binding capacity of Fab_α fragments opens the interesting possibility that IgA1 protease-producing bacteria growing in the presence of IgA1 antibodies are coated with Fab_α fragments. Such fragments may conceivably protect the microorganism from the immune system by blocking access of intact antibody molecules of the same or other isotypes and of immunocompetent cells. A similar strategy for escaping the host immune system has been described for certain protozoa (1, 2, 4). If this hypothesis is correct, the question of whether cleavage of IgA can keep step with secretion of intact antibodies becomes irrelevant. It would, furthermore, explain why specific cleavage of IgA in the hinge region may be a more efficient way of escaping the immune system than extensive degradation induced by nonspecific proteases.

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