Working Mechanism of Immunoglobulin A1 (IgA1) Protease: Cleavage of IgA1 Antibody to Neisseria meningitidis PorA Requires De Novo Synthesis of IgA1 Protease

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Neisseria meningitidis secretes a protease that specifically cleaves the hinge region of immunoglobulin A1 (IgA1), releasing the effector (Fc) domain of IgA1 from the antigen binding (Fab) determinants. Theoretically, the remaining Fab fragments can block pathogen receptors or toxins and still provide protection. Here, we describe binding of V-gene-matched human IgA1 and IgA2 to PorA of strain H44/76. On live meningococci, efficient cleavage of IgA1, but not cleavage of IgA2, was observed, and up to ~80% of the IgA1 Fc tails were lost from the meningococcal surface within 30 min. No cleavage of IgA1 was found on an isogenic H44/76 strain lacking IgA protease. Furthermore, our data indicate that PorA-bound IgA1 is masked by the serogroup B polysaccharide capsule, rendering the IgA1 less accessible to degradation by secreted IgA1 protease present in the bacterial surroundings. Experiments with protein synthesis inhibitors showed that de novo production of IgA1 protease was responsible for cleavage of PorA-bound IgA1 on encapsulated bacteria. Finally, our data suggest that cleavage of IgA1 by IgA1 protease releases a significant proportion of Fab fragments from the bacterium, probably as a result of their reduced avidity compared to that of whole antibodies.

One of the hallmarks of adaptive immunity is the generation of antigen-specific antibodies upon pathogen recognition. For mucosal commensals and pathogens, this predominantly evokes production of locally produced immunoglobulin A (IgA) antibodies; in humans these antibodies are secretory IgA1 (sIgA1) or sIgA2 antibodies. Approximately 90% of the available upper respiratory IgA formed in humans is the IgA1 subclass (5, 9). At mucosal surfaces, IgA is believed to inhibit microbial adhesion to host cells, to neutralize microbial toxins and enzymes, and to interact with IgA receptor-bearing phagocytes and thereby eliminate invading pathogens (13). Based on the local production and high concentrations of IgA in respiratory mucosa, IgA-mediated immunity is thought to provide a first line of defense against recurrent mucosal pathogens.

Some mucosa-restricted bacteria, including gram-negative bacteria as well as gram-positive bacteria, seem to have acquired countermechanisms by convergent evolution, involving secretion of a protease that specifically cleaves human IgA1 in its hinge region and does not cleave not IgA2 or other immunoglobulins (8). This results in separation of the two antigen-binding Fab fragments from the Fc tail, which is responsible for cross-linking and activating phagocyte IgA Fc receptors (13, 28), suggesting that there is a functional way in which the IgA1 proteases protect bacteria from the host immune system. The remaining Fab fragments are generally believed to be capable of conferring some level of protection against bacteria, as they may still bind to bacterial surfaces and thereby block interactions between bacterial proteins and human target structures. Conversely, IgA1 protease-producing bacteria may take advantage of released Fab fragments by enhancing the surface hydrophobicity and thus adhesion (32) and by blocking the access of intact antibodies (8).

Some of the IgA1 protease-producing bacteria are the human-restricted pathogens Streptococcus pneumoniae, Neisseria meningitidis, Neisseria gonorrhoeae, and Haemophilus influenzae (8). The gram-negative species N. meningitidis, N. gonorrhoeae, and H. influenzae all produce a homologous serine protease (8), while the gram-positive pneumococcus produces a functionally homologous, but evolutionary distinct metalloprotease (16, 30). It has been suggested that the secreted form of these proteases is responsible for cleaving mucosal IgA1 antibodies already bound to bacteria, as well as unbound sIgA1 molecules in their surroundings (8, 9). Consequently, this may change the balance between pathogen elimination and survival in favor of the invader. Solid experimental evidence for the precise mechanisms and biological significance is, however, lacking.

N. meningitidis remains the most common cause of bacterial meningitis, especially in infants and teenagers (18). While efforts to generate protective vaccines have been successful for some capsular serogroups, serogroup B requires an alternative vaccine strategy since antigenic determinants of its polysialic acid capsule are found in the developing brain (6). Protective antibodies directed against exposed surface proteins are found in sera of convalescent patients (31) but are also formed upon colonization (33). A major proportion of these antibodies are directed against porin A (PorA) (31, 33).

Neisserial IgA1 protease cleaves the hinge region of both...
FIG. 1. Generation of an IgA1 protease knockout construct. The domain organization in the iga gene is shown at the top. The corresponding domains are labeled, and the active site is indicated by an asterisk. A 1,100-bp fragment of the 5′ part of the gene was amplified with primers CF and CB, and an EcoRI site was fitted in the gene fragment by site-directed mutagenesis. A kanamycin resistance cassette was cloned into the resulting EcoRI site, and the resulting construct was used to transform H44/476 meningococci. Primers KF and igB were used to verify that insertion into the H44/476 genome took place by homologous recombination.

serum and secretory IgA1 (1), and recent findings have identified other targets of neisserial IgA1 proteases (7, 10, 19). In this study, we used human V-gene-matched human IgA1 and IgA2 antibodies directed against PorA of N. meningitidis (28) together with meningococcal strain H44/76 and an isogenic H44/76 strain lacking IgA1 protease expression to study the degradation of IgA antibodies on the surface of live bacteria.

MATERIALS AND METHODS

Bacteria. The H44/76 strains of N. meningitidis (B:15:P1.7,16) were stored in Mueller-Hinton broth (NV1, Bihloven, The Netherlands) with 20% glycerol at −70°C. Isogenic uncapsulated HB-1 mutated strains were generated as described previously (25, 26). The day before use, bacteria were plated onto GC agar plates (Becton Dickinson, San Jose, CA) and incubated overnight at 37°C in the presence of 5% CO2. Bacteria were then grown in Mueller-Hinton broth from an optical density at 620 nm (OD620) of 0.08 to an OD620 of 0.220 (duration, 1.5 h) for experiments to assess the proteolytic degradation of IgA. For experiments involving expression of IgA1 protease, H44/76 was grown in tryptic soy broth (Difco, Detroit, MI) from an OD620 of 0.1 to an OD620 of 1.5.

IgA1 protease-deficient strain. A general outline of the methods used to generate an IgA1 protease-deficient strain is shown in Fig. 1. As the iga gene from H44/76 had not been completely sequenced, we selected primers in the leader peptide (GCCGCGCTAAATACGGCCTACATCTCC) and DNA uptake sequence underlined [4] and 3′ of the active site (GCCCTGAAAACACG ACC) based on conserved sequences in other meningococcal iga genes described previously (strains MC58, Z2491, and Ng44/76, with accession numbers ACC) with similar affinities (22, 23). The generation, specificity, and production of these antibodies have been described in detail previously (28). Anti-1ga (α chain specific, IgA1 and IgA2) and anti-human-kappa light chain antisera were purchased from Southern Biotechnology Associates (Birmingham, AL) as phycoerythrin (PE)-labeled goat F(ab)2 fragments. An antisera to IgA1 protease was raised in rabbits against a recombinant version of the HF13 protease described by Lomholt et al. (11).

Expression of IgA1 protease. One milliliter of an H44/76 culture was centrifuged at 2,200 × g for 5 min. The bacterial pellet was frozen at −20°C, and the supernatant was precipitated with 20% trichloroacetic acid at −20°C overnight. The precipitate was centrifuged at 20,000 × g for another 20 min. The remaining protein pellet was resuspended in 20 μl phosphate-buffered saline, and 5 μl of sample buffer (3% [wt/vol] Tris, 50% [vol/vol] glycerol, 10% [wt/vol] sodium dodecyl sulfate [SDS], 10% [wt/vol] dithiothreitol, 0.05% bromophenol blue) was added. The frozen bacterial pellets were thawed and resuspended in 50 μl phosphate-buffered saline, and then 10 μl sample buffer was added. Supernatants (8 μl) and 6-μl portions of the protein pellets were analyzed on 11% SDS-polyacrylamide gel electrophoresis gels, using a Protein II minigel system (Bio-Rad, Veenendaal, The Netherlands). One gel was stained with Coomassie blue G250 stain (Bio-Rad), and another gel was blotted onto a 0.2-μm Protran filter (Schleicher & Schuell) using a semidyed electroblotter (Ancos, Hoqby, Denmark) at 100 V for 1 h.

Anti-IgA1 protease serum was diluted 1:500 in washing solution (0.01 M Tris, 0.154 M NaCl, 0.5% Tween 20). After washing, blots were incubated with goat anti-rabbit IgG serum conjugated to alkaline phosphatase (Southern Biotechnology Associates) diluted 1:5,000 in washing solution with 0.5% Protifar (Nutricia, Zoetermeer, The Netherlands). Binding was then visualized with nitroblue tetrazolium-BCIP (5-bromo-4-chloro-3-indolylphosphate) (Bio-Rad).

Proteolytic degradation of IgA. H44/76 group B meningococci (107 CFU) was washed two times with RPMI 1640 (Life Technologies, Gaithersburg, MD) with 5% fetal calf serum (Life Technologies) and incubated with 2 μg/ml IgA1 or IgA2 (unless indicated otherwise) in the presence of bacterial supernatants in a 250-μl total volume mixture. The bacteria were incubated with immunoglobulins for 45 min at 37°C, washed twice with RPMI 1640 to remove unbound IgA, and then incubated for 30 min at 37°C in the presence of bacterial supernatants (end log phase) or RPMI 1640 (250 μl). When indicated below, chloramphenicol (10 μg/ml) was present throughout the incubations. Subsequently, bacteria were heat killed by incubation for 30 min at 56°C, and IgA bound to bacteria was quantified with either a chain-specific anti-IgA or anti-human kappa light chain serum. In all experiments, the fluorescence of bacteria was determined for 10,000 bacteria by fluorescence-activated cell sorting (FACS) analyses with a FACSCalibur (Becton Dickinson). Loss of IgA activity found on bacterial surfaces (percent IgA degradation) was calculated as follows: [GM for IgA1 or IgA2 on ΔGM − GM for IgA1 on test sample]/GM for IgA1 on ΔGM × 100%, where GM is the geometric mean.

Statistical analyses. A one-tailed paired t test was performed using GraphPad Prism, version 4.00 for Windows (GraphPad Software, San Diego, CA) (http://www.graphpad.com), to test our hypothesis that IgA1 is degraded on the surface of wild-type H44/76 compared to igA-deficient H44/76. The level of significance was defined as P < 0.05.

RESULTS

Disruption of the H44/76 iga gene results in loss of IgA1 protease expression. Kanamycin-resistant H44/76 was analyzed for correct insertion of a knockout construct and production of IgA1 protease. Using a kanamycin-specific primer (primer KF) (Fig. 1) and a primer specific for the iga gene but 3′ of the primer used for cloning of the original knockout construct (primer igB) (Fig. 1), we obtained a band at the predicted size, ~1,500 bp, for only the mutated strain (Fig. 2A). Whole cells and bacterial supernatants were then analyzed for expression of IgA1 protease. While no (or very little) expression of IgA1 protease was detectable in whole cells, high protein levels were found in supernatants of wild-type H44/76 bacteria but not in iga-deficient H44/76 bacteria (Fig. 2B). As...
previously reported (15, 26), two bands corresponding to the protease domain (apparent molecular weight, 110,000) and the protease domain with the alpha peptide (apparent molecular weight, 160,000) (Fig. 2) were observed in the supernatant of the wild-type H44/76 bacteria.

Release of Fc tails and Fab fragments after opsonization of H44/76 with human IgA1 but not after opsonization with IgA2. Encapsulated H44/76 and iga-deficient bacteria were opsonized with chimeric V-gene-matched human IgA1 and IgA2 anti-PorA antibodies (28). The levels of opsonization for the two IgA subclasses on iga-deficient H44/76 were indistinguishable (Fig. 3B), whereas the IgA1 levels were decreased by 65% on WT H44/76 (Fig. 3A). The loss of IgA reactivity of WT H44/76 compared to iga-deficient bacteria proved to be greater with short incubation times with IgA1 (Fig. 3C). The loss of IgA1 reactivity of the iga-deficient strain was compatible with an average doubling time of 23 min after incubation. Interestingly, significant dissociation of Fab fragments from the bacterial surfaces was consistently observed (Fig. 4).

Effect of bacterial supernatants on IgA opsonization. To establish whether the secreted IgA protease of H44/76 contributes to the cleavage of IgA1 bound to PorA, opsonized WT or iga-deficient bacteria were incubated in the presence of meningococcal culture supernatants with and without IgA1 protease obtained from end-log-phase WT H44/76 and iga-deficient meningococci, respectively (Fig. 4). Virtually no increase in IgA1 proteolytic activity was observed when WT H44/76 was incubated with supernatants from H44/76 compared to the activity with supernatants from H44/76 iga-deficient meningococci. However, significant degradation was observed with IgA1-opsonized H44/76 iga-deficient bacteria in the presence of IgA1 protease-containing H44/76 supernatants, albeit to a lesser extent than when WT H44/76 was used (Fig. 4, compare bars 1 and 3).

Requirement of de novo protein synthesis for optimal IgA1 protease activity. H44/76 was incubated in the presence of IgA protease knockout construct results in gene inactivation and lack of expression. (A) Genomic PCR using primers KF and igaB resulted in a ~1,500-bp fragment in the iga-deficient H44/76 strain but not in its wild-type counterpart, indicating that there was successful insertion into the iga gene. (B) Expression of IgA1 protease in H44/76 as shown by Coomassie brilliant blue staining (lanes 1 to 4) and Western blot analyses (lanes 6 to 9). Whole-cell lysates (WC) (lanes 1, 2, 6, and 7), as well as supernatants (Sup) (lanes 3, 4, 8, and 9), were analyzed on H44/76 (1, 3, 6, and 8) and its isogenic iga-deficient strain (2, 4, 7, and 9). The arrows indicate the positions of the 110-kDa and 160-kDa forms of IgA1 protease. Experiments were repeated two times, with similar results.

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chloramphenicol at a concentration that was sufficient to block protein synthesis, as judged by growth inhibition (data not shown), but low enough to allow survival of bacteria, as judged by growth resumption when chloramphenicol was removed. The bacteria were then opsonized as described above with IgA1 and IgA2 antibodies. IgA1 was not degraded on the surface of H44/76 after chloramphenicol treatment since it was detected at levels similar to the levels of IgA2 (Fig. 5A and B). Incubation with bacterial supernatants of end-log-phase H44/76 resulted in a minimal effect on IgA1 opsonization on H44/76 in the presence of chloramphenicol (Fig. 5C), whereas supernatants of iga-deficient H44/76 had no effect (Fig. 5D).

Capsule protects IgA1 bound to PorA from degradation by IgA1 protease. Once secreted, the IgA1 protease seemed to be less able to cleave PorA-bound IgA1 antibodies. To test whether the capsule of H44/76 interfered with the accessibility of IgA1 protease to PorA-bound IgA1, an unencapsulated variant of H44/76, HB-1, and an iga-deficient derivative of this variant were opsonized with IgA1 antibodies and incubated with WT or iga-deficient H44/76 supernatants. IgA1 was equally degraded on cells of H44/76 and its capsule-deficient H44/76 had no effect (Fig. 5D).

FIG. 4. Secre ted IgA1 protease present in meningococcal supernatants contributes little to overall IgA1 protease activity that also results in release of bound Fab fragments from H44/76. WT H44/76 or iga-deficient bacteria were incubated with IgA1 together with end-log-phase medium from WT or iga-deficient H44/76. IgA1 oposinization of neisseriae was determined by FACS analyses and is expressed as the percent IgA1 or kappa light chains released from bacteria compared to the data for the H44/76 iga-deficient strain with iga-deficient supernatants. Oposinization levels were detected with PE-labeled antibodies to IgA (α-chain specific) and to kappa light chains to detect meningococcus-associated IgA and Fab fragments, respectively. The data are means and standard deviations from four experiments, all of which produced similar results. The lines above the graph summarize the results of statistical comparisons with the leftmost bars indicated (one asterisk, \( P < 0.01 \); two asterisks, \( P < 0.001 \); NS, not significant).

FIG. 5. Chloramphenicol blocks degradation of IgA1 bound to PorA. IgA1 oposinization was determined by flow cytometry using anti-IgA-PE on untreated H44/76 meningococci (A) or in the presence of chloramphenicol (B to D). H44/76 meningococci were incubated with RPMI 1640 medium (B), H44/76 supernatant (C), or H44/76 iga-deficient supernatant (D). Gray histograms, IgA1; solid lines, IgA2; dashed lines, negative control. Experiments were repeated three times, with essentially identical results.

FIG. 6. Capsule-deficient (HB-1) H44/76 and \( \Delta \)iga H44/76 HB-1 were opsonized with 5 μg/ml IgA1 in the presence or absence of IgA1 protease-containing WT supernatants. IgA1 oposinization was determined with PE-labeled anti-IgA antibodies as described in the legend to Fig. 4. The data are means and standard deviations from three experiments in which similar results were obtained. The results of statistical comparisons are indicated as described in the legend to Fig. 4.
unencapsulated bacteria, this value rose to ~90% of the maximal degradation (Fig. 6, compare bars 5 and 7). Taken together, IgA1 bound to PorA on the capsule-deficient Δiga strain was significantly more sensitive to degradation by IgA1 protease in supernatants than its capsule proficient counterpart, which indicated that the capsule protects PorA-bound IgA1 from proteolytic degradation by secreted IgA1 protease.

**DISCUSSION**

For the most part, the mechanism of IgA1 protease cleavage of IgA1 has been studied in vitro using concentrated IgA1 protease preparations from culture broths incubated together with immunoglobulin preparations, followed by SDS-polyacrylamide gel electrophoresis analyses to detect fragmentation of IgA1 (1, 3, 9). Thus, these studies simulated how the secreted IgA1 protease cleaves (nonspecific) IgA1 in mucosal secretions. In most, if not all, of these studies, concentrated preparations of IgA1 proteases were used and/or incubations took place for a long time (hours).

In the present study we took a different approach, using V-gene-matched IgA1 and IgA2 antibodies recognizing the same PorA epitope located in loop 4, whose binding and functional properties have been studied in detail (22, 23, 28). We studied IgA1 cleavage on the surface of live meningococci by comparing the levels of deposited IgA1 and IgA2 on WT strain H44/76 and its iga-deficient derivative using FACS analyses. To our knowledge, a study of IgA1 degradation by IgA1 proteases on the surface of intact bacteria has never been described before. Binding of IgA1 and IgA2 to the surface of iga-deficient H44/76 was comparable, indicating that the observed degradation of IgA1 on WT H44/76 meningococci, and not degradation of IgA2, was attributable to expression of the IgA1 protease gene.

In *N. meningitidis* and *N. gonorrhoeae*, the iga gene encodes a serine protease belonging to the autotransporter family (15). The nascent polypeptide chain is secreted first into the periplasm. There, the autotransporter domain forms a β-barrel pore in the outer membrane (14, 15, 27) through which the passenger domain (IgA1 protease domain) (Fig. 1) reaches the cell surface. The protease domain is then autocleaved from the transporter domain and released into the medium. The apparent fast kinetics of this process made it impossible for us to demonstrate the presence of IgA1 protease on the neisserial H44/76 surface by immunolabeling analyses (data not shown) (n = 2). Accordingly, we could detect only significant amounts of IgA1 protease in supernatants (Fig. 2). These data are in accordance with previous reports (15, 17), and, therefore, it has been assumed that most of the proteolytic activity toward anti-meningococcal IgA1 is represented by the IgA1 protease after secretion.

Surprisingly, supernatants of H44/76 expressing IgA1 protease mediated only moderate cleavage of IgA1 bound on the surface of heat-killed H44/76 (data not shown) and on live iga-deficient H44/76 or WT H44/76 treated with chloramphenicol (Fig. 4 and 5). This suggested that the majority of IgA1 bound to PorA is cleaved by IgA1 protease before its eventual release and not by the secreted form present in supernatants. We argue that a possible explanation for this phenomenon is that the bacterial surface is rendered less accessible to soluble proteins by the polysaccharide capsule. Indeed, when unencapsulated Δiga H44/76 HB-1 strain and its encapsulated Δiga derivative were incubated with supernatants containing IgA1 protease, the PorA-bound IgA1 proved to be much more sensitive to degradation by IgA1 protease on unencapsulated meningococci (Fig. 6). Similar mechanisms, possibly involving steric hindrance by the capsule and/or the size, shape, and charge of the protein entering the capsule, have been implicated in the lower accessibility of serum proteins for subcapsular antigens on various *S. pneumoniae* serotypes (2, 20) and serogroup B meningococci (12). Although our data suggested that IgA1 protease is somewhat (but not completely) restricted from reentering the capsule, they do not support the hypothesis that IgA1 has fewer restrictions to enter the capsule. However, the two proteins are likely to differ fundamentally in the ability to exit the capsule, as movement of IgA1 protease is likely to be governed only by the laws of Brownian motion, whereas the IgA1 used in this study was antigen specific and bound firmly to PorA. The detection antisera [PE-labeled F(ab')2] may also be restricted from entering the capsule in a manner similar to that of IgA. However, F(ab')2 is significantly smaller than whole IgA and may therefore be less restricted than whole immunoglobulin. Furthermore, degradation was defined as the amount of IgA1 detected on capsule-matched WT H44/76 compared to iga-deficient H44/76. Our results therefore suggest that PorA-bound IgA1 is cleaved in the (partially protected) environment of the capsule, by either transiently surface-bound or recently released IgA1 protease.

The cleavage site of the neisserial IgA1 protease is N terminal to that of cysteine residues that covalently link the two immunoglobulin heavy chains. After cleavage of IgA1 the two resulting Fab fragments have lower avidity compared to divalent and covalently linked F(ab')2 fragments or whole IgA1. Depending on the affinity of the antibody, Fab fragments are expected to retain antigen binding (high-affinity antibodies) or loose detectable reactivity (low-affinity antibodies). The chimeric IgA molecules bind to the loop 4 epitope like the mouse IgG2a antibody from which they were derived with moderate affinity ($K_a = 1.4 \times 10^{-8}$) (21, 28). These data are compatible with our observations that Fab fragments of papain-digested IgG1 (V gene...
matched to the IgA molecules described here [28] exhibit minor binding to H44/76 compared to that of the F(ab)2 fragments of pepsin-digested IgG1, which have binding indistinguishable from that of untreated IgG1 (unpublished results).

Accordingly, we document here that IgA1 Fc fragments not only seem to be released from the meningococcal surface but also seem to be part of the corresponding Fab fragments. We consistently observed that the reduction in Fab fragment binding of WT strain H44/76 was similar, albeit always slightly lower than, the reduction observed for whole IgA1 bound to PorA. This indicates that proteolytic cleavage of this IgA1 results in formation of Fab fragments with severely affected PorA-binding capability, which are released from the bacterial surface.

Taken together, as summarized in Fig. 7, our data suggest that de novo production of neisserial IgA1 protease represents the major source of degradation of IgA1 already bound to PorA on the surface of encapsulated bacteria, releasing the effector Fc domain of IgA1 from the bacteria. The remaining monovalent Fab fragments exhibited diminished antigen-binding capacity. IgA1 protease expression can therefore help Neisseria evade opsonization not only by eliminating the presence of Fc fragments of IgA1 already bound but also by reducing the neutralizing ability of IgA1 antibodies by reducing their binding avidity.

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