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Production of Immunoglobulin A Protease by *Streptococcus pneumoniae* from Animals

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Human isolates of *Streptococcus pneumoniae* tested by traditional immunochemical methods produce a protease that cleaves human immunoglobulin A1 (IgA1) into Fab and Fc fragments. The protease may be an important virulence factor, but studies of its pathogenetic significance have been hampered by lack of a suitable animal model. Since *S. pneumoniae* is a respiratory pathogen for several species of animals, we sought to determine whether isolates of this organism from animals with pneumococcal infection, including fatal diplococcal pneumonia, produced an IgA protease. Isolates from six animal species including the mouse, rat, dog, guinea pig, rhesus monkey, and chimpanzee were tested for protease activity against IgA preparations from the mouse, rat, dog, guinea pig, rabbit, rhesus and cynomolgus monkeys, gorilla, and human. Cleavage of IgA was demonstrated by the appearance of Fc fragments in Western blots (immunoblots) treated with specific antisera. All these isolates except that from the guinea pig produced a protease that cleaved IgA of human, rhesus monkey, and gorilla origin. Cleavage was inhibited by 5 mM EDTA. IgA cleavage from the other species could not be demonstrated. Although *S. pneumoniae* can colonize the respiratory tracts of several animal species, it is a significant pathogen principally of humans and some other primates. Our data suggest that some species of nonhuman primates including the rhesus monkey could be suitable for experimental studies on the significance of IgA protease in the pathogenesis of pneumococcal disease.

Various studies estimate that 30 to 70% of the human population is infected with *Streptococcus pneumoniae* (9, 10, 17, 18). Most infected persons are asymptomatic carriers, but the organism is also an important primary cause of pneumonia, meningitis, bronchitis, sinusitis, and otitis media (9, 18). To date, virtually all isolates of *S. pneumoniae* tested by immunoelectrophoretic or autoradiographic techniques have been shown to produce a protease that cleaves immunoglobulin A1 (IgA1) into Fab and Fc fragments (19–21, 28, 34, 35, 37). Bacterial IgA proteases are presumed to play a significant role in enabling organisms producing them, including *S. pneumoniae* and the gonococcus, to colonize mucosae and perhaps potentiate their invasion of other tissues and organs (24, 34–37). Experimental verification of this postulate has been hampered by lack of an animal model. Proteases studied to date have been from human isolates of *S. pneumoniae* and have been shown to cleave only human IgA1. Other human and animal serum proteins, including immunoglobulins, are not hydrolyzed (19, 20, 24, 28, 34, 35). The substrate specificity of the protease for human IgA1 may simply reflect that isolates tested to date have been of human origin. *S. pneumoniae* is not a strict human pathogen; it is known to also colonize the nasopharynx and cause respiratory disease in several animal species (2–5, 11–13, 16, 20, 33, 38, 39, 41). The occurrence of pneumococcal infections and disease in several species other than humans suggested the possibility of the production of other host-specific IgA proteases. We therefore sought to determine whether isolates of *S. pneumoniae* from animals with either inapparent infection or fatal pneumococcal pneumonia produced an IgA protease and to determine the activity of the protease against human IgA and IgA preparations from several animal species.

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MATERIALS AND METHODS

Bacteria. *S. pneumoniae* (ATCC 27336) and *Escherichia coli* (ATCC 25922) were used as human IgA1 protease-positive and -negative controls, respectively. Animal isolates of *S. pneumoniae* (Table 1) were identified by optochin sensitivity, bile solubility (27), and capsular serotyping (performed by the University of Minnesota Hospital microbiology laboratory). Isolates were subcultured weekly on tryptic soy agar with 3% sheep erythrocytes (DiMed, St. Paul, Minn.), incubated at 35°C for 24 h, and stored at room temperature.

Immunoglobulins and antisera. Human serum IgA (Organon Teknika, Westchester, Pa.) was dissolved in distilled water (0.5 mg/ml) and stored in 0.1-ml aliquots at −48°C. Secretory IgA from the dog, guinea pig, mouse, rabbit, and rat was prepared from colostrum obtained by hand milking animals twice daily for the first 3 days postpartum. Suckling mice and rats were euthanatized by cervical dislocation at 3 days of age, and colostrum from their stomachs was pooled with mammary gland colostrum. Colostrum was diluted with an equal volume of 0.85% NaCl solution and centrifuged at 9,681 × *g* for 1 h at 8°C. The clear middle layer was removed with a pipette, dialyzed (Spectropore membrane; molecular weight cutoff, 12,000 to 14000; Terminal Annex, Los Angeles, Calif.) with stirring at 8°C for 1 h in Tris-saline buffer (0.5 M Tris, 0.5 M NaCl, pH 7.7), and centrifuged again at 9,681 × *g* for 30 min at 8°C. The supernatant was dialyzed in Tris-saline buffer for 16 to 18 h at 8°C, sterilized by filtration (Acrodisc; 0.02-μm pore size; Gelman Sciences, Inc., Ann Arbor, Mich.), and stored in 0.1-ml aliquots at −48°C. Proteins in serum samples from the gorilla, rhesus monkey, and cynomolgus monkey were precipitated with 50% ammonium sulfate and centrifuged at 9,651 × *g* for 30 min at 8°C.

TABLE 1. *S. pneumoniae* isolates used for IgA protease activity assays

Organism	Source	Species of origin	Culture site	Capsular serotype
S. pneumoniae	University of Minnesota, Veterinary Diagnostic Laboratories	Dog	Nares	3
S. pneumoniae	Animal Disease Diagnostic Laboratories, University of Missouri, Columbia	Guinea pig	Pharynx	19
S. pneumoniae	Division of Comparative Medicine, Massachusetts Institute of Technology, Cambridge	Mouse	Lung	3
S. pneumoniae	Division of Animal Medicine, University of Washington, Seattle	Rat	Lung	19
S. pneumoniae	Division of Animal Medicine, University of Washington, Seattle	Rat	Lung	35
S. pneumoniae	Delta Primate Center, Covington, La.	Rhesus monkey	Lung	19
S. pneumoniae	Delta Primate Center, Covington, La.	Rhesus monkey	Lung	19
S. pneumoniae	Yerkes Primate Center, Atlanta, Ga.	Chimpanzee	Lung	3
S. pneumoniae[a]	ATCC 27336	Human	NT[c]	NT
E. coli[b]	ATCC 25922	Human	NA[d]	NA

[a] Positive control.
[b] Negative control.
[c] NT, Nontypeable.
[d] NA, Not applicable.

The precipitate was dissolved in 1.0 ml of phosphate-buffered saline (PBS) (0.85% NaCl, 20 mM $NaHPO_4$, 0.1% thimerosol, pH 7.1.), dialyzed in PBS with stirring for 1 h at 8°C, and then diluted in an equal volume of PBS. IgG was removed by protein A-Sepharose chromatography (4 by 10 cm column; Sigma Chemical Co., St. Louis, Mo.) (26, 30). The IgA-enriched preparation in the void volume was eluted with PBS, whereas IgG was eluted with citrate-phosphate buffer (0.1 M citric acid, 0.2 M Na_2HPO_4, pH 4.5) and dialyzed in PBS (30). Western blots (immunoblots) of IgA-enriched preparations from the gorilla, rhesus monkey, and cynomolgus monkey were unreactive to peroxidase-conjugated protein A and were thus essentially free of IgG. Similarly, the IgG fractions from these nonhuman primates were nonreactive with anti-human IgA but were strongly reactive with peroxidase-conjugated protein A and anti-rhesus monkey IgG in Western blots.

Peroxidase-conjugated antisera (IgG, Fc specific) of goat origin for human, mouse and rat IgA and unconjugated antisera (IgG, Fc specific) of goat origin for dog IgA and rhesus monkey IgG and sheep origin for guinea pig IgA were obtained from Nordic Immunologic Laboratories (Capistrano Beach, Calif.). Unconjugated goat antiserum to rabbit secretory IgA was purchased from Organon Teknika, Cappel Division. The lyophilized antisera were dissolved in distilled water at 5.0 mg/ml and stored in 0.05-ml aliquots at −48°C. Purified IgG of human, mouse, rat, and rhesus monkey origin (Sigma) was reconstituted in distilled water (1 mg/ml) and stored at −48°C. Antiserum specificity and the presence of IgA in preparations of sera or milk were determined by immunoelectrophoresis (14) or Western blotting (7). The IgA antisera were unreactive with IgG from the homologous species and also did not react with light chains. Antiserum to human IgA was used to detect gorilla, rhesus monkey, and cynomolgus monkey IgA (40). Western blots of the IgA-enriched preparations from the gorilla, rhesus monkey, and cynomolgus monkey did not react with monkey IgG antiserum, but IgG from these species in the citrate buffer eluate from protein A-Sepharose columns reacted strongly with monkey IgG antiserum.

IgA protease assay. Bacteria were cultured at 35°C for 24 h on tryptic soy agar with 3% sheep erythrocytes. Growth was removed with a sterile cotton swab and suspended in distilled water to an approximate concentration of 1.2×10^9 bacteria per ml (27). Equal volumes of fresh bacterial suspension and IgA-containing solution were added to individual wells of polystyrene tissue culture plates (Microtest III; Becton Dickinson and Co., Lincoln Park, N.J.). The plates were covered tightly with Parafilm and incubated at 35°C for 24 h. The mixture was aspirated from each well with a Pasteur pipette and centrifuged at 15,600 × *g* for 5 min, and the supernatant was stored in 0.1-ml aliquots at −48°C until subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Control mixtures of IgA with distilled water or with *E. coli* were treated similarly. The viability of the bacteria was determined before and after each assay by culture. Inhibition of IgA protease activity by the metal chelator sodium EDTA (34) was evaluated by the addition of 5 mM EDTA to mixtures of organisms and IgA solutions before incubation and electrophoresis.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed with vertical slab gels (5% stacking, 10% running) as described by Laemmli (25). Samples were mixed with an equal volume of SDS buffer (10% glycerol, 5% 2-mercaptoethanol, 3.3% SDS, 0.3% Tris, 0.5 M NaCl, 0.001% bromophenol blue, pH 7.7) and heated at 100°C for 5 min. Electrophoresis was done at 25 mA for approximately 5 h, after which the gels were either stained with Coomassie blue (15) or subjected to protein blotting (7). After electrotransfer, nitrocellulose sheets were immersed in blocking solution (2% nonfat dry milk in distilled water) with continuous mixing on a rocking platform for 30 min at room temperature. Appropriate IgA antiserum was added, and the solution was rocked for 1 h. The sheets were then washed for 30 min in two changes of PBS. The incubation and washing procedures were repeated with a protein A-peroxidase conjugate in blocking solution when unconjugated antiserum was used. The transferred proteins were visualized by the addition of substrate solution (0.05% 3,3-diaminobenzidine, 0.1% H_2O_2, 0.048 M citric acid, 0.05 M sodium citrate) immediately after the final wash. Intact IgA and Fc fragments were identified with Fc-specific antisera (30, 31) and molecular mass markers (SDS-6H; Sigma). Western blots of nonhuman primate IgA preparations demonstrating cleavage were also reacted with peroxidase-conjugated protein A to determine whether cleaved products might represent IgG fragments. These blots were unreactive with protein A.

RESULTS

Eight isolates of *S. pneumoniae* from six species of animals were tested for production of IgA protease. Seven of

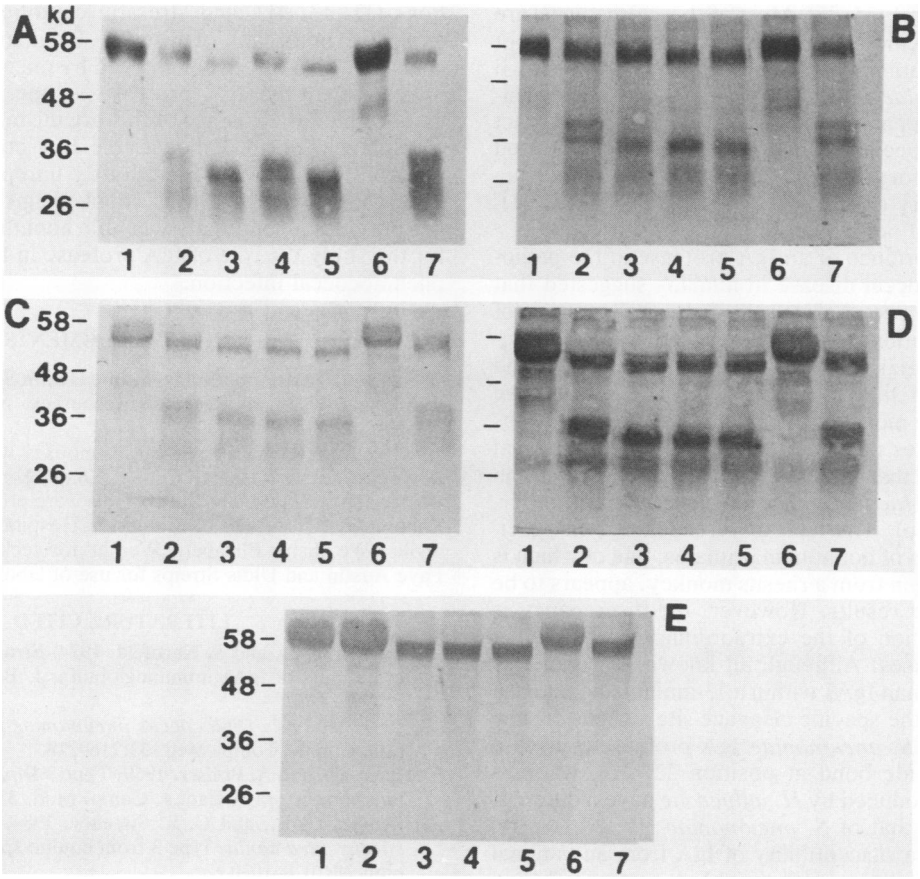

FIG. 1. Western blots demonstrating IgA protease activity of isolates of *S. pneumoniae* on IgA preparations from human serum (A), human colostrum (B), rhesus monkey serum (C), gorilla serum (D), and cynomolgus monkey serum (E). The species of origin of the *S. pneumoniae* isolates were as follows: lanes 1, negative control; lanes 2, human; lanes 3, mouse; lanes 4, rat; lanes 5, dog; lanes 6, guinea pig; lanes 7, rhesus monkey. The blots were developed with goat Fc-specific antisera to human IgA. IgA preparations from the mouse, rat, dog, guinea pig, and rabbit were also incubated with these isolates of *S. pneumoniae* and developed with appropriate Fc-specific antisera. Western blots of these samples revealed that IgA was not cleaved and appeared essentially similar to panel E. kd, kilodaltons.

the eight isolates produced an IgA protease, but cleavage could be demonstrated only for IgA from the human, gorilla, and rhesus monkey. Cleavage was not observed in IgA samples from the mouse, rat, dog, guinea pig, rabbit, and cynomolgus monkey. IgA protease activity could not be demonstrated for the guinea pig isolate.

Western blots demonstrating cleavage of IgA from the human, gorilla, and rhesus monkey after incubation with isolates of *S. pneumoniae* from several animal species are shown in Fig. 1A through D (lanes 2 through 5 and 7). The heavy-chain-specific antisera usually reacted with up to three bands in samples in which IgA was cleaved. The predominant banding pattern varied with the species and whether the source of IgA was serum or milk. Cleaved IgA from human and nonhuman primate sera usually produced two dark bands representing uncleaved heavy chains, including IgA2 heavy chains at the origin (~55 kilodaltons) and Fc fragments (~32 to 36 kilodaltons). A third and less distinct band in the 28- to 30-kilodalton region (Fig. 1A to D) was interpreted to represent other heavy-chain fragments with different molecular sizes produced by the variable action of pneumococcal glycosidases (8, 19, 23, 34, 37), and possibly the heavy-chain portion of the Fab fragment of IgA1. Differences in the relative positions of bands probably reflect interspecies variation in protein composition and glycosylation of IgA and the extent of carbohydrate degradation by bacterial enzymes (1, 8, 20, 23, 32, 37). Inclusion of EDTA in the IgA protease assay mixture inhibited protease activity such that Western blots of IgA preparations from the rhesus monkey and human that contained 5 mM EDTA during incubation lacked Fc fragments and appeared identical to the immunoblot shown in Fig. 1E.

In contrast to results obtained with samples from the human, gorilla, and rhesus monkey, samples from the mouse, rat, dog, guinea pig, rabbit, and cynomolgus monkey showed no cleavage of IgA after incubation with *S. pneumoniae*. A typical immunoblot from a nonsusceptible species is exemplified by the IgA preparation from the cynomolgus monkey in Fig. 1E. The lack of IgA protease production by the guinea pig isolate is shown in Fig. 1A through D, lanes 6.

Additional experiments were done to determine whether protease inhibitors might account for lack of IgA cleavage. Human serum IgA was added to each sample of animal IgA that was resistant to cleavage. Western blots of these samples were exposed sequentially to the anti-species and anti-human IgA antisera. In each instance, the human IgA was cleaved, thereby indicating an absence of inhibitors.

DISCUSSION

IgA proteases have been implicated as potentially important virulence factors produced by a variety of human

bacterial pathogens (24, 34, 37). Most of these organisms are known to be pathogenic for only human beings and do not cause significant naturally occurring diseases in other animal species. *S. pneumoniae* is an exception because this organism resides as an inapparent infection in the respiratory tract of several animal species (4, 11, 38, 41) and has caused epizootics of respiratory disease, meningitis, and septicemia in common laboratory animals (2, 3, 12, 22, 29, 33, 38, 41), livestock (4, 38), and nonhuman primates (5, 13, 16, 39).

The putative importance of an IgA protease in the pathogenesis of pneumococcal disease in humans suggested that this protease plays an equivalent role in the pathogenesis of *S. pneumoniae* infections in animals. One report (24) mentioned that a bacterial IgA protease cleaved gorilla and chimpanzee IgA but that the same protease was inactive against IgA from the mouse, rat, rabbit, dog, and 20 species of nonhuman primates not otherwise identified. The animal IgA preparations in that study (24) were tested against an IgA protease from *Haemophilus influenzae* (A. Plaut, personal communication). The lack of IgA cleavage in specimens from 20 species of nonhuman primates, one of which is presumed to have been from a rhesus monkey, appears to be at variance with our results. However, the discrepancy is most likely a reflection of the extraordinary specificity of bacterial IgA proteases. Although all known bacterial IgA proteases cleave human IgA1 within a 13-amino-acid interval in the hinge region, the specific cleavage site varies with the species of bacteria. *S. pneumoniae* IgA protease cleaves a prolyl-threonyl peptide bond at position 227-228, whereas the IgA proteases produced by *H. influenzae* have a different site specificity than that of *S. pneumoniae* (19, 20, 28, 34, 37). Accordingly, the susceptibility of IgA from any animal species to cleavage by known bacterial IgA proteases would depend on the degree of amino acid homology within the hinge region to that of human IgA. Before an IgA substrate could be classified as being resistant to cleavage, it would have to be tested against the several IgA proteases with different amino acid bond and site specificities. It follows therefore that cynomolgus monkey IgA, although resistant to cleavage by *S. pneumoniae* IgA protease, may be susceptible to cleavage by other bacterial IgA proteases.

The lack of IgA protease production by the guinea pig isolate of *S. pneumoniae* appears to be unusual because virtually all isolates studied to date have produced the enzyme (19–21, 24, 28, 34, 35, 37). IgA protease deficiency in this isolate may reflect a genetic mutation similar to that described in some isolates of *H. influenzae* (6). This protease-deficient isolate may prove to be useful in comparative studies on the pathogenesis of pneumococcal disease with protease-positive and protease-negative variants.

These results contribute to a mounting body of evidence indicating that the IgA protease produced by *S. pneumoniae* cleaves only IgA1 of human origin and IgA (presumably IgA1) from some nonhuman primates. Whereas previous studies demonstrated these findings with human isolates of the diplococcus (19, 20, 28, 35), the study described here used isolates from animals with naturally occurring inapparent infection or clinical disease. The specificity of the protease for primate IgA even among protease-producing isolates from nonprimate species and the substantially higher prevalence of pneumococcal infection and disease in human beings relative to other animal species suggest that animal isolates of this organism are primarily of human origin. In many instances, infections in animals are largely incidental and inapparent. However, transmission of the organism from animal to animal occurs readily under colony conditions (11, 13, 41), and stressful conditions can precipitate explosive outbreaks of disease. Fatal infections in animal species whose IgA is not cleaved by pneumococcal protease indicate that the IgA protease is one of several factors responsible for virulence of this organism. We also identified an isolate with no demonstrable IgA protease activity, an apparently unusual and previously unreported observation. The susceptibility of rhesus monkey IgA to protease cleavage provides a potentially valuable nonhuman primate model for the study the role of IgA protease in the pathogenesis of pneumococcal infection.

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