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Loss of Antibody Activity in Human Immunoglobulin A Exposed to Extracellular Immunoglobulin A Proteases of *Neisseria gonorrhoeae* and *Streptococcus sanguis*

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Immunoglobulin A (IgA) proteases are extracellular enzymes elaborated by *Neisseria gonorrhoeae*, *N. meningitidis*, and *Streptococcus sanguis*. These enzymes each cleave human IgA1 at a critically situated prolyl-threonyl peptide bond to yield Fabα and Fcα fragments. To study their effect on the antibody activity of human IgA, we enzymatically digested a group of five human IgA monoclonal immunoglobulins with high-titer rheumatoid factor or cold agglutinin activity and human serum macroamylase, an amylase-IgA complex. In contrast to four control IgM rheumatoid factor monoclonal proteins, whose activity was unaffected by enzyme, gonococcal and streptococcal IgA proteases caused prompt, major reductions of IgA antibody activity to negligible levels and converted macroamylase activity to amylase of normal size, as determined by molecular sieve chromatography. In addition, both enzymes promptly deagglutinated sensitized cells that had been aggregated by IgA rheumatoid factors, indicating that IgA bound to antigen is also susceptible to enzyme cleavage. Fab fragments of IgA protein Chr, a rheumatoid factor, showed essentially no antigen-binding activity despite the high titers observed with the parent protein. These studies emphasize the high degree of specificity of the microbial proteases for IgA and their potential for interfering with antibody activity in the IgA1 subclass.

Immunoglobulin A (IgA) proteases are extracellular enzymes of *Streptococcus sanguis*, *Neisseria gonorrhoeae*, and *N. meningitidis* that cleave IgA proteins of the IgA1 subclass to yield Fabα and Fcα fragments (10, 12, 13). These enzymes cleave IgA1 protein by attacking prolyl-threonyl peptide bonds in the "hinge region" of the heavy chain. Human proteins of the IgA2 subclass are IgA protease resistant because they have a short but critically located deletion in the primary structure of the heavy chain, which includes the enzyme-susceptible peptide bonds. Both the streptococcal and gonococcal IgA proteases are incapable of cleaving a large number of proteins other than human IgA, including the IgA of other species and all other classes of human immunoglobulins. The extreme specificity of these enzymes for human IgA suggests that they may be important in infection, since the pathogens that release these enzymes enter at mucosal sites defended by a local secretory immune system mediated largely by IgA antibody (8, 15). For this reason, we have undertaken experiments to determine the effect of these microbial enzymes on human antibody activity of the IgA class.

The unambiguous assignment of a given antibody to IgA in sera or secretions of human beings is difficult because allegedly pure preparations of normal IgA are frequently contaminated by IgG or IgM when examined by sensitive techniques. To surmount this problem we determined the effect of IgA protease on a group of human IgA myeloma proteins specifically selected because they have such high titers of antibody that reduction in titers can only be ascribed to changes in the IgA and not to contaminants of other immunoglobulin classes. In addition, we examined the effect of IgA protease on macroamylase (2, 9), an amylase-IgA antibody complex that accumulates in the sera of patients with liver disease because its large size prevents glomerular filtration.

MATERIALS AND METHODS

Nine human sera containing homogeneous immunoglobulin populations with antibody activity were studied. Five sera contained IgA proteins;

three of these had rheumatoid factor activity in that they showed specificity for human IgG, and two had cold agglutinin activity for human or sheep erythrocyte antigens. The paraproteins were all assigned to the IgA1 subclass based on their precipitation in double-diffusion agar gels with rabbit anti-human IgA1 antiserum. This antiserum was prepared by absorbing an anti-human IgA serum with a pool of purified human IgA2 subclass proteins. No attempt was made to assess the degree of polymerization of these monoclonal proteins. The other four sera were from patients with Waldenstrom macroglobulinemia, and all contained monoclonal IgM proteins with rheumatoid factor activity at a high titer. The antibody titers of these sera in our laboratory are shown in Table 1. Considering the rarity of homogeneous human immunoglobulins having antibody activity, we are indebted to a number of individuals who generously made these sera available for our use. By convention, the three-letter code word used to identify the proteins comes from the first three letters in the last name of the patient providing the serum.

Streptococcal IgA protease enzyme was purified from the bacteria-free supernatant fluid drawn from 24-h cultures of *S. sanguis* (American Type Culture Collection no. 10556) grown in Todd-Hewitt broth as described previously (11). IgA protease of *N. gonorrhoeae* was partially purified from culture filtrates of a Kellogg colony type 2 organism grown in Difco GC broth supplemented with IsoVitaleX (12). Both enzyme preparations were highly active against human IgA, cleaving these proteins to Fcα and Fabα fragments as determined by electrophoretic and immunologic analysis of the digestion products (10, 13). Although precise units for enzyme activity are not yet available, the enzyme preparation from each microorganism, when incubated at 37°C with an equal volume of a solution of IgA at 10 mg/ml (pH 7.2), was able to cleave all the IgA substrate in approximately 60 min as determined by electrophoresis on cellulose acetate (13).

In the present studies IgA protease treatment of sera or purified proteins was done in a water bath at 37°C in 0.05 M tris(hydroxymethyl)aminomethane buffer, pH 8.1. The enzyme preparations were added in equal volume with the protein or serum under study, and incubation was carried out for 2 h. Enzymatic activity was stopped by the addition of ethylenediaminetetraacetate to a final concentration of 50 mM.

To prepare immunoglobulin fragments of protein Chr, the purified IgA myeloma protein was digested with *S. sanguis* IgA protease. The degree of hydrolysis with resultant fragment generation was followed by immunoelectrophoresis (14). Fragments were purified chromatographically at pH 8.1 in non-dissociating conditions on molecular sieve columns (Bio-Gel P-200, Bio-Rad Laboratories, Richmond, Calif.) and were characterized immunologically with anti-immunoglobulin antisera of established specificity. The characterization of Fabα and Fcα fragments and details of their separation are described in detail in earlier publications (10, 13).

Extrinsic labeling of protein Chr with ^{125}I was done at pH 7.5 by the chloramine-T method of Hunter and Greenwood (7). Unbound ^{125}I was removed by column chromatography on Sephadex G-50. Radioactive isotope counting was done on a Beckman Biogamma II counter (Beckman Instruments Co., Fullerton, Calif.).

Rheumatoid factor titers were determined by both slide agglutination of globulin-coated latex particles (Ra-Test, Hyland Division, Travenol Laboratories, Costa Mesa, Calif.) and hemagglutination with sheep erythrocytes (SRBC) (Colorado Serum Com-

TABLE 1. *Influence of streptococcal IgA protease on antibody activity*[a]

Serum	Paraprotein class	Test system	Reciprocal titer			Effect of enzyme on antibody activity
			Initially	After IgA protease treatment	After heated IgA protease treatment	
Chr	IgA	Latex-IgG	5,120	40	5,120	Marked decrease
		SRBC-IgG	20,480	320	20,480	Marked decrease
Dun	IgA	Latex-IgG	5,120	20	5,120	Marked decrease
Boy	IgA	SRBC-IgG	1,280	80	1,280	Marked decrease
Fin	IgA	SRBC	163,840	0	163,840	Marked decrease
Rob	IgA	Human RBC[b]	5,000,000	20,480	5,000,000	Marked decrease
Kas	IgM	Latex-IgG	3,000	3,000	3,000	No effect
Lay	IgM	SRBC-IgG 4°C	4,096	4,096	4,096	No effect
Lat	IgM	Latex-IgG	6,000	6,000	6,000	No effect
Fag	IgM	Latex-IgG	6,000	6,000	6,000	No effect
Rheumatoid arthritis		Latex-IgG	800	800	800	No effect
		SRBC-IgG	5,120	2,560	5,120	Minor reduction

[a] Antibody activity of homogeneous proteins in several human sera. None of the sera studied contained inhibitor to either enzyme. The data show a marked reduction in titer of IgA antibodies and essentially no change in IgM antibodies, a result consistent with the in vitro susceptibility of IgA alone to enzyme cleavage.

[b] RBC, Erythrocytes.

pany Laboratories, Denver, Colo.) sensitized in our laboratory with heat-aggregated human Cohn fraction II (IgG) by the chromium chloride method (4). Hemagglutination titers were determined in microtiter hemagglutination plates (Cooke Engineering Co., Alexandria, Va.) by serial twofold dilutions of antibody in a suspension of sensitized SRBC. Plates were studied for cell agglutination after 1 h. Cold agglutinin studies were performed with human group B erythrocytes (Ortho Diagnostic Inc., Raritan, N.J.) or SRBC preserved in Alsiever solution but washed in buffer before use. Hemagglutination plates were incubated for 2 h at 5°C before they were read.

To determine if IgA antibodies would be enzymatically cleaved once they were bound to antigen, IgG-SRBC agglutinated by the IgA sera Chr and Dun and by the IgM sera Kas and Lat were used. For these studies, erythrocytes were agglutinated by undiluted portions of each of the four proteins mentioned. The dense agglutinates were lying in the form of a granular film at the bottom of microtiter wells containing 0.2 ml of tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 8.1. To each well was added 0.025 ml of streptococcal or gonococcal IgA protease, and incubation was undertaken at 37°C (in an air incubator), during which time the pellet was examined at 5-min intervals for deagglutination. Control wells received buffer instead of enzyme. Deagglutination was defined as conversion of the observed agglutinate to a typical, sharply defined erythrocyte "button" of the type seen in the absence of hemagglutination.

Human serum containing macroamylase was from a 46-year-old man with micronodular cirrhosis of the liver related to alcohol abuse. The amylase level in the serum was 1,289 Somogyi units per 100 ml (normal 50–150), and a 2-h urine collection on the same day showed 11 U. The amylase activity in this serum was known to be bound to IgA by earlier studies, which had shown precipitation of nearly all of the amylase by rabbit anti-human IgA antiserum under conditions described by Levitt and Cooperband (9). To test the capacity of IgA proteases to change the molecular size of this amylase, equal volumes of IgA protease enzyme and serum at pH 8.1 were incubated for 1 h at 37°C. The digests were subsequently fractionated on molecular sieve columns, and eluates were assayed for amylase activity by the Phadebas amylase test (Pharmacia Laboratories, Inc., Piscataway, N.J.).

RESULTS

Incubation of the antigen-binding IgA proteins with both streptococcal and gonococcal IgA proteases resulted in pronounced reduction in antibody activity as shown in Tables 1 and 2. In contrast, no significant reduction in titer was observed when the four monoclonal IgM rheumatoid factors were treated with the same enzyme preparations under identical conditions. Similarly, polyclonal rheumatoid factor activity in the sera of rheumatoid arthritis patients was not significantly affected by enzyme treatment.

To determine if IgA protease enzyme was active against IgA antibody already bound to antigen, IgG-SRBC agglutinated by IgA sera Chr and Dun were treated with streptococcal and gonococcal IgA proteases at 37°C as described in the text. In contrast to the undisturbed agglutination in control incubations, exposure to either enzyme caused complete deagglutination of cells easily visible after 5 min of digestion at 37°C. Because this occurred with

TABLE 2. *Influence of gonococcal IgA protease on antibody activity*[a]

Serum	Paraprotein class	Test system	Reciprocal titer			Effect of enzyme on antibody activity
			Initially	After IgA protease treatment	After heated IgA protease treatment	
Chr	IgA	Latex-IgG	5,120	0	5,120	Marked decrease
		SRBC-IgG	20,480	160	20,480	Marked decrease
Dun	IgA	Latex-IgG	5,120	20	5,120	Marked decrease
Boy	IgA	SRBC-IgG	1,280	0	1,280	Marked decrease
Fin	IgA	SRBC	163,840	20	163,840	Marked decrease
Rob	IgA	Human RBC[b]	5,000,000	5,000	5,000,000	Marked decrease
Kas	IgM	Latex-IgG	3,000	3,000	3,000	No effect
Lay	IgM	SRBC-Ig 4°C	4,096	4,096	4,096	No effect
Lat	IgM	Latex-IgG	6,000	6,000	6,000	No effect
Fag	IgM	Latex-IgG	6,000	6,000	6,000	No effect
Rheumatoid arthritis		Latex-IgG	800	800	800	No effect
		SRBC-IgG	5,120	5,120	5,120	No effect

[a] Antibody activity of homogeneous proteins in several human sera. As in the case of the streptococcal IgA protease (Table 1), the antibody titers of IgA protein alone were adversely affected, whereas IgM titers were unchanged.

[b] RBC, Erythrocytes.

the IgA proteins at their lowest dilution (most concentrated), the titer of the Chr and Dun proteins had essentially fallen to zero. Enzymatic deagglutination of cells aggregated with IgM sera Kas and Lat did not occur, reflecting the specificity of the enzymes for the IgA class. IgA protease-deagglutinated cells, washed at 4°C with tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.1) to remove enzyme and solubilized IgA antibody fragments, were readily reagglutinated by addition of the fresh IgA sera, indicating that antigenic sites were not completely blocked by the Fabα fragment or other products of digestion. The failure of the digestion products of proteins Dun and Chr to block antigen binding by fresh antibody suggested that the Fabα fragment was not capable of antigen binding. This led to further experiments with the Chr protein, which was available in sufficient quantity to study in greater detail.

Protein Chr, a protein studied in detail by Abraham et al. (1), was purified from plasma by their method, and the resultant product was adjusted to a rheumatoid factor titer of 1:20,000. The IgA was free of IgG and other serum protein contamination as determined by Ouchterlony double-diffusion analysis with well-characterized rabbit and goat antisera to human serum proteins. For purposes of obtaining fragments, 5.0 mg of the IgA was labeled with ^{125}I and digested at 37°C for 16 h with streptococcal enzyme. The Fcα and Fabα fragments were separated on Bio-Gel P-200 at pH 8.1 (10) and adjusted to a 1-mg/ml protein concentration, and the labeled fragments were tested for binding to IgG-SRBC. SRBC sensitized by purified human serum transferrin served as a control for nonspecific binding. As shown in Table 3, neither Chr Fabα nor Fcα fragments bound significantly to IgG-SRBC, since isotope counts were only 10% those using intact Chr IgA at a concentration of 1 mg/ml and were equivalent to nonspecific fragment binding to control transferrin-SRBC.

IgA protease treatment of the serum that contained macroamylase caused a distinct change in the size of the amylase as determined by elution position on a Bio-Gel P-100 column (Fig. 1). Before IgA protease treatment, ap-

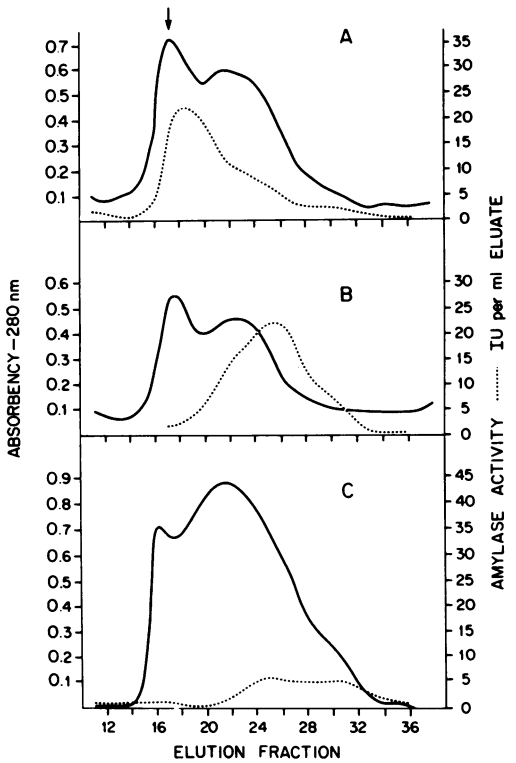

FIG. 1. *Reduction in size of macroamylase by treatment with streptococcal IgA protease, as determined by chromatography in Bio-Gel P-100. (A) Macroamylasemic serum. Amylase activity under dotted line elutes just after void volume (↓) and before the broad albumin peak. (B) Serum as in A after IgA protease treatment. Amylase now elutes much later, after the albumin peak, in a position corresponding to that of normal serum amylase as seen in (C). Incubation of the macroamylasemic serum with boiled, inactive IgA protease did not change amylase size.*

TABLE 3. *Capacity of CHR IgA and its fragments to bind to IgG-sensitized SRBC*[a]

Protein or fragment examined	Binding to IgG-SRBC			Binding to transferrin-SRBC		
	Total counts added	Counts bound	Counts bound (%)	Total counts added	Counts bound	Counts bound (%)
^{125}I-labeled Chr IgA	60,991	21,451	35.2	63,215	2,615	4.14
^{125}I-labeled Chr Fcα	80,106	2,526	3.15	79,676	2,636	3.3
^{125}I-labeled Chr Fabα	40,307	437	1.08	44,565	345	0.7
^{125}I-labeled human transferrin	138,690	4,686	3.37	ND	ND	ND

[a] Experiment showing loss of capacity of Chr Fcα and Fabα fragments to bind to IgG-sensitized SRBC when compared with intact Chr IgA. Transferrin-sensitized SRBC served as a control of nonspecific binding. ND, Not determined.

proximately 90% of the amylase activity in this serum eluted near the void volume of the column, indicating that it had a molecular weight exceeding that of normal serum amylase (molecular weight, 44,000). Normal serum amylase eluted from the same column after the marker albumin peak, indicating that it had the expected molecular weight of approximately 44,000. After 60 min of exposure of the macroamylase serum to either gonococcal or streptococcal IgA protease, rechromatography revealed that all amylase activity could now be found in the "normal" elution position corresponding to that of normal serum amylase. A portion of the same serum that had been incubated with heat-inactivated IgA protease as a control showed no shift in amylase position in the same molecular sieve column. The amylase in normal serum was unchanged in size when exposed to IgA protease. These data indicate that both the gonococcal and streptococcal enzymes markedly reduced the molecular weight of the amylase in a macroamylase complex involving IgA, whereas normal serum amylase was unaltered by exposure to enzyme.

DISCUSSION

The data presented here indicated that microbial IgA proteases derived from *S. sanguis* and *N. gonorrhoeae* greatly reduce or abolish effective antibody activity of the IgA class by an enzymatic mechanism. Our decision to focus on the rare homogeneous IgA proteins with antibody activity in these studies was to take advantage of their high titer, allowing us to exclude the possibility that contaminating IgG or other immunoglobulin classes contributed to the antibody activity under study in these preparations. We believe that the gross reduction in titer after treatment with IgA protease from both microbial species establishes the point that IgA antibody is highly sensitive to cleavage by these enzymes. The inability of these enzymes to influence the antibody activity of homogeneous IgM proteins is consistent with the insusceptibility of human IgM in vitro to cleavage by both of the IgA proteases. It should be noted that antibody activity in secretory IgA was not examined in these experiments.

The prompt enzymatic conversion of macroamylase to amylase of essentially normal size in the serum examined correlates well with earlier detailed work from other laboratories showing that IgA is the protein to which amylase is frequently bound in such sera (9). Since IgA protease apparently cleaves only the human IgA1 subclass, results in the example studied indicate that the IgA to which the amylase is bound is of the IgA1 subclass. The use of the microbial IgA proteases as reagents to further examine the interesting phenomenon of macroamylasemia or other biological or structural characteristics of IgA is suggested by this work.

Although the role that these enzymes have in initiating or perpetuating bacterial pathogenesis is not known, their capacity to cleave bound as well as free antibody of the IgA1 subclass shows their potential ability to frustrate the function of IgA at several levels. The finding that digestive fragments of proteins Chr and Dun failed to inhibit the agglutination of IgG-SRBC by the intact proteins indicates that the Fabα fragments of these proteins are unable to bind effectively to antigen. This is corroborated by those experiments showing that purified monovalent ^{125}I-labeled Chr Fabα fragments do not attach to cells. These data underscore the principle that a structurally intact Fab fragment derived from antibody may have a very low functional affinity, although it embodies the antigen-binding site in unaltered form. This emphasizes the key role played by polyvalency in observed antibody efficiency (3). By using anti-2,4-dintrophenyl antibodies in rabbits, Hornick and Karush (6) noted that the functional affinity of bivalent IgG antibody is 10^3-fold greater than the intrinsic affinity of the monomer, whereas IgM anti-2,4-dinitrophenyl, as anticipated from its higher valence, has a 10^6-fold-greater functional affinity compared with its monomer. These differences can be attributed to multivalency of antibody, and they emphasize the biologic importance of immunoglobulin polymerization. In the case of IgA, both the serum and secretory forms have a natural tendency to polymerize, and the main product of the secretory immune system is dimeric IgA, a tetravalent antibody protein (15). Thus, despite the fact that enzymatic hydrolysis by IgA protease leaves an intact Fabα, functional antibody activity may be severely compromised in cleaved antibody populations.

As emphasized in earlier reports (12, 13), since both the gonococcal and streptococcal enzymes cleave only the IgA1 subclass of human IgA, they would, by inference, be ineffective in reducing the antibody activity in a population of IgA2 subclass proteins. Whereas we would like to verify this directly, we have been unable to identify a human IgA2 subclass homogeneous protein having antibody activity to a known antigen. With the exception of human colostrum (5), the relative concentrations of IgA1 and IgA2 proteins at mucosal surfaces have not been studied in enough detail to evaluate the role of IgA proteases in bacterial infection. The present data, however, indicate the

potential that these enzymes have for interfering with IgA antibody activity.

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