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Molecular Aspects of Immunoglobulin A1 Degradation by Oral Streptococci

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Using a panel of 143 strains classified according to a novel taxonomic system for oral viridans-type streptococci, we reexamined the ability of oral streptococci to attack human immunoglobulin A1 (IgA1) molecules with IgA1 protease or glycosidases. IgA1 protease production was an exclusive property of all strains belonging to *Streptococcus sanguis* and *Streptococcus oralis* (previously *S. mitior*) and of some strains of *Streptococcus mitis* biovar 1. These are all dominant initiators of dental plaque formation. Degradation of the carbohydrate moiety of IgA1 molecules accompanied IgA1 protease activity in *S. oralis* and protease-producing strains of *S. mitis* biovar 1. Neuraminidase and β-galactosidase were identified as extracellular enzymes in organisms of these taxa. By examination with enzyme-neutralizing antisera, four distinct IgA1 proteases were detected in *S. sanguis* biovars 1 to 3, *S. sanguis* biovar 4, *S. oralis*, and strains of *S. mitis*, respectively. The cleavage of IgA1 molecules by streptococcal IgA proteases was found to be influenced by their state of glycosylation. Treatment of IgA1 with bacterial (including streptococcal) neuraminidase increased susceptibility to protease, suggesting a cooperative activity of streptococcal IgA1 protease and neuraminidase. In contrast, a decrease in susceptibility was observed after extensive deglycosylation of the hinge region with endo-α-*N* acetylgalactosaminidase. The effector functions of IgA antibodies depend on the carbohydrate-containing Fc portion. Hence, the observation that oral streptococci may cleave not only the α1 chains but also the carbohydrate moiety of IgA1 molecules suggests that the ability to evade secretory immune mechanisms may contribute to the successful establishment of these bacteria in the oral cavity.

Streptococci of the viridans type are constant and numerically significant members of the indigenous oral flora. Apart from being an occasional cause of subacute endocarditis, oral streptococci are considered nonpathogenic. However, this group of bacteria is of significance in relation to dental disease because of an ability to successfully colonize hard and soft oral surfaces. Streptococci belonging to the species *Streptococcus mitis*, *Streptococcus oralis* (previously *S. mitior*), and *Streptococcus sanguis* are dominant among the bacteria that colonize a newly cleaned tooth surface (30), thereby initiating the formation of dental plaque, which may eventually cause caries and periodontal disease.

The successful establishment of oral streptococci might be explained not only by their adherent properties, but also by an ability to evade local defense mechanisms. On mucosal surfaces, specific immunological defense is mediated primarily by secretory immunoglobulin A (S-IgA). Besides, by its ability to neutralize toxins and prevent the penetration of antigenic substances, S-IgA contributes to the protection of mucosal surfaces by reducing microbial colonization (17). S-IgA is considerably more resistant to proteolytic attack than other immunoglobulin classes. In view of this, it is interesting that a number of bacteria indigenous to the oral cavity, including *S. sanguis* and *S. oralis* as well as oral *Bacteroides* and *Capnocytophaga* species, have been shown to release IgA1 protease (14, 15). IgA1 proteases are endopeptidases characterized by their specificity for one of several post-proline peptide bonds located within a sequence of 13 amino acids present in the hinge region of human IgA molecules belonging to the A1 subclass (19). In accordance with the fact that this sequence is deleted in the α2 heavy chain of IgA2 molecules, this subclass is resistant to cleavage. As a consequence of IgA1 protease activity, S-IgA1 molecules are left as antigen-binding Fab fragments devoid of the Fc_nSC part, which is particularly responsible for the effector functions of S-IgA antibodies (17). IgA1 proteases are also produced by a number of overt pathogens, including *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Neisseria meningitidis*, and *Neisseria gonorrhoeae*, and there is convincing support for the hypothesis that in these species IgA1 proteases are important virulence factors (19). Data recently reviewed (20) indicate that IgA1 protease activity may be of significance also for the apparent ability of oral streptococci to withstand the effects of the secretory immune system.

In addition to the release of IgA1 proteases, some oral and nonoral bacteria have been shown to produce extracellular glycosidases able to cleave the carbohydrate moiety of IgA1 molecules (7, 16). The functional significance of these enzymes is not known.

The taxonomy of oral viridans-type streptococci has recently been thoroughly revised. Resulting changes include subdivision of *S. sanguis* into *S. sanguis* sensu stricto and *Streptococcus gordonii* sp. nov. and a redefinition of *S. oralis* ("*S. mitior*") and *S. mitis* (18). The present study reexamined the ability of these and other oral streptococci to degrade human IgA1 with IgA1 protease or glycosidases and compared the IgA1 proteases from the individual species with regard to enzyme specificity and inhibition by neutralizing antibodies. Furthermore, the interaction between glycosidase- and IgA protease-induced degradation of the IgA1 substrate was investigated.

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TABLE 1. IgA1 degradation induced by oral streptococcal species

Species	No. of strains examined	No. of strains revealing indicated type of IgA1 degradation		
		IgA1 protease (pattern 1)	IgA1 protease + carbohydrate depletion (pattern 2)	Carbohydrate depletion[a] (pattern 3)
S. sanguis	31	31	0	0
S. gordonii	23	0	0	0
S. oralis	20	0	20	0
S. mitis biovar 1	10	0	2	0
S. mitis biovar 2	13	0	0	8
S. salivarius	12	0	0	0
S. anginosus	24	0	0	0
S. mutans	10	0	0	0

[a] Carbohydrate depletion; see Table 2.

MATERIALS AND METHODS

Bacterial strains. The study included 143 streptococcal strains that had been subjected to detailed phenotypic and serological characterization as part of a taxonomic study on viridans-type streptococci (18). The strains, which included several type and reference strains obtained from national collections, were assigned to the species *S. sanguis*, *Streptococcus gordonii* sp. nov., *S. oralis*, *S. mitis* biovars 1 and 2, *Streptococcus salivarius*, *Streptococcus anginosus*, and *Streptococcus mutans* (Table 1) according to the principles described by Kilian et al. (18).

Preparation of extracellular bacterial enzymes. Crude preparations of IgA1 proteases and extracellular glycosidases from selected streptococcal strains were made by ammonium sulfate precipitation (60%, pH 7.2) of supernatants of 24-h cultures in Todd-Hewitt broth. The redissolved precipitates were cleared by centrifugation, concentrated five to ten times by negative pressure filtration, and dialyzed against 0.01 M phosphate-buffered saline, pH 7.4, containing 0.04% sodium azide (PBS).

IgA1 proteases from *S. sanguis* ATCC 10556 and *S. oralis* ATCC 10557 were purified by size-exclusion chromatography of crude enzyme preparations on a column (1.6 by 50 cm) of Superose 12 (Pharmacia, Uppsala, Sweden) equilibrated in 0.1 M phosphate buffer, pH 7.0, containing 0.04% sodium azide. Proteins were eluted with the same buffer at 1 ml/min. The column had been calibrated for molecular weight determination by use of the following standards: α_2-macroglobulin (720,000), dimeric serum IgA (320,000), IgG (150,000), bovine serum albumin (68,000), ovalbumin (45,000), and myoglobin (12,200). IgA1 protease-containing eluent fractions were identified by their capacity to cleave myeloma IgA1 as measured in an assay based on enzyme immunoassay principles (described below). Eluent fractions were furthermore monitored for neuraminidase and β-galactosidase activity with chromogenic substrates. Neuraminidase was detected by the method of Potier et al. (34) by its capacity to cleave 4-methylumbilliferyl-α-D-*N*-acetylneuraminate. Free 4-methylumbilliferone was detected in a Shimadzu fluorescence spectrophotometer operated at excitation and emission wavelengths of 365 and 450 nm, respectively. For the detection of β-galactosidase, 20 μl of eluent fractions was incubated overnight at 35°C with 100 μl of 1% *O*-nitrophenyl-β-D-galactopyranoside (Sigma Chemical Co., St. Louis, Mo.) in 0.067 M phosphate buffer, pH 8.0. After the addition of 50 μl of 2 M NaOH, cleaved substrate was measured spectrophotometrically at 405 nm.

IgA substrates. Myeloma IgA1 proteins (Fri, Mor, and Kah) were purified from the serum of patients with multiple myeloma as described previously (39). The preparations of Fri and Mor were purely monomeric, whereas the Kah preparation consisted of dimeric IgA1 molecules. IgA1 (Fri) had been used in previous studies of IgA1 proteases from strains of *Haemophilus influenzae*. From the electrophoretic mobilities of Fab and Fc fragments produced by two strains cleaving the peptide bonds at positions 231-232 and 235-236, respectively, it was evident that the span between the two cleavage sites contained charged carbohydrate residues, i.e., sialic acid. For use in certain experiments, myeloma IgA1 proteins were subjected to three different glycosidase treatments. One treatment was with neuraminidase from *Vibrio cholerae* (Behringwerke, Marburg, Federal Republic of Germany [FRG]). A solution of IgA1 protein (3 mg/ml) was dialyzed against 0.05 M sodium acetate buffer, pH 5.5, containing 0.15 M NaCl and 0.009 M CaCl. Neuraminidase was added in an amount of 0.1 U per ml of IgA1 solution. After incubation at 35°C for 18 h, the protein was redialyzed against PBS. Another sample of IgA1 was treated with a crude preparation of extracellular enzymes from *S. mitis* biovar 2 strain SK96 containing neuraminidase and β-galactosidase activity. One volume of IgA1 solution (3 mg/ml in PBS) was incubated with 0.1 volume of crude enzymes at 35°C for 18 h and then redialyzed against PBS. The third form of carbohydrate-deprived IgA1 resulted from treatment with *V. cholerae* neuraminidase (as described above) followed by endo-α-*N*-acetylgalactosaminidase from *S. pneumoniae* (Boehringer Mannheim, Mannheim, FRG). The latter enzyme liberates the disaccharide Gal(β1-3)GalNAc, O linked to serine or threonine core units, and was added in an amount of 20 mU/ml after dialysis of the neuraminidase-treated substrate against 0.1 M sodium cacodylate buffer, pH 6.0. After incubation at 35°C for 18 h, the substrate was redialyzed against PBS. Corresponding to each of the glycosidase-treated substrates, a sham-treated substrate was produced by parallel incubation and dialysis procedures but without the use of glycosidases. The effect of each treatment was examined by quantitative carbohydrate analysis of enzyme-treated and sham-treated substrates (see below).

Detection of IgA-degrading enzyme activity by electrophoresis. The IgA1-degrading activity of streptococcal strains was demonstrated by mixing a loopful of bacteria from a 24-h blood-agar culture or 20 μl of extracellular enzyme preparation with 40 μl of a 2-mg/ml solution of IgA1 in PBS. After incubation for 18 to 24 h at 35°C, IgA1 degradation was revealed by immunoelectrophoresis (IEP) and/or sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). IEP was done in 2% Noble agar in Veronal buffer, pH 8.6. The antisera used to develop the IEP were our own rabbit serum raised against purified colostral S-IgA and rabbit antibodies specific for heavy (α) and appropriate light chains of IgA (Dakopatts, Glostrup, Denmark). For analysis by SDS-PAGE, samples of enzyme-treated IgA1 were reduced and fractionated in 4 to 20% polyacrylamide density gradient gels as described previously (39).

Examination of IgA1 proteases with enzyme-neutralizing antibodies. Female white rabbits, weighing 3 to 4 kg, were immunized by subcutaneous injections of 0.5 ml of crude IgA1 protease preparations mixed with 0.5 ml of Freund incomplete adjuvant at intervals of 2 weeks until a satisfactory titer of protease-neutralizing antibodies was obtained. The immunoglobulin fraction of the sera was isolated as

described by Harboe and Ingild (13). As no inhibitory activity was ever obtained by immunizing with *S. oralis* IgA1 protease, a human serum from a patient with subacute endocarditis due to *S. oralis* was used instead. The serum was a gift from E. Gutschik, Copenhagen. The titer of enzyme-neutralizing antibodies was defined as described previously (21).

Inhibition of bacterial neuraminidase. In some experiments the activity of streptococcal neuraminidase was inhibited by addition of 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (Böhringer, Mannheim, FRG) (21). Before the addition of IgA1 substrate, 4 volumes of extracellular streptococcal enzymes were incubated for 1 h at room temperature with 1 volume of the neuraminidase inhibitor dissolved at 5 mM in 0.2 M Tris-acetate buffer, pH 7.2. Pilot experiments revealed that complete inhibition of streptococcal neuraminidase was obtained at this concentration of the inhibitor.

Quantitative analysis of IgA1 carbohydrates. The effect of streptococcal strains on the carbohydrate moiety of IgA1 substrate was examined. Ten milligrams (dry weight) of bacteria harvested from an agar plate and washed three times in PBS were incubated with 2 mg of myeloma IgA1 in 1 ml of PBS for 24 h at 35°C. Samples in which IgA or bacteria were omitted were coincubated as controls. Bacteria were then removed by centrifugation (10,000 × *g* for 30 min), and the samples were exhaustively dialyzed against redistilled water. Monosaccharides from IgA were determined as trifluoroacetates of methylglycosides by gas-liquid chromatography. Neutral sugars and sialic acid were liberated by incubation of the glycoprotein with freshly prepared 0.5 M HCl in anhydrous methanol at 80°C for 24 h as described before (46). Aminohexoses were determined following hydrolysis with aqueous 4 N HCl for 4 h at 100°C. After evaporation of the excess of HCl in vacuo, the samples were subjected to methanolysis as described above. Aminohexoses were then N-acetylated and partially de-O-acetylated (36). Monosaccharides were quantitated relative to internal standards (2 nmol of sorbitol), which was added after methanolysis. Derivatization and gas chromatography were performed as described before (47). The protein content of samples was determined by the method of Bradford (3) with bovine serum albumin as the standard. The effect obtained by treatment of IgA1 substrate with different preparations of glycosidases was examined by the same procedure.

Determinations of cleavage site. The peptide bond in the IgA1 molecule cleaved by the IgA1 protease of *S. oralis* ATCC 10557 was determined by partial N-terminal amino acid sequencing of Fc_α fragments purified from the substrate IgA1 (Mor) after cleavage. The methods used for purification of Fc_α and amino acid sequencing have been described (28).

Experiments with glycosidase-treated IgA1 substrates. The effects of three different pretreatments with glycosidase (described above) on the susceptibility of IgA1 myeloma proteins to degradation by IgA1 proteases from *S. sanguis* ATCC 10556 and *S. oralis* ATCC 10557 were examined. Portions (200 μl) of IgA1 myeloma proteins (0.1 mg/ml in PBS) subjected to the individual treatments and similar portions of corresponding sham-treated control substrates were incubated in parallel at 35°C with 100 μl of partly purified streptococcal IgA1 protease. The neuraminidase activity present in the protease preparation from *S. oralis* ATCC 10557 was inhibited with 2 mM 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid. At regular intervals during the incubation, 25-μl samples were withdrawn from the reaction mixtures and added to 25 μl of 100 mM EDTA to inhibit IgA1 protease activity. At the end of the incubation period, all samples withdrawn were examined in parallel for IgA1 protease-induced cleavage by an enzyme-linked immunosorbent assay (ELISA) described previously (37). Briefly, the samples were diluted in PBS containing 20 mM EDTA and 0.15% Tween 20 to an IgA1 concentration of 1 μg/ml and added, in triplicate, in micro-ELISA wells coated with a mouse monoclonal antibody specific for the Fc portion of the α-heavy chain (Dakopatts; clone BE2C1). The monoclonal antibody had been bound to the well by a catching layer of rabbit antibody specific for mouse immunoglobulins (Dakopatts). After incubation and washing, Fab portions retained in the wells as part of uncleaved or incompletely cleaved IgA1 molecules were allowed to react with peroxidase-conjugated rabbit antibody specific for light chains of the type carried by the IgA1 substrate (Dakopatts). Following another wash, wells were incubated with *O*-phenylene diamine–H_2O_2 solution and read spectrophotometrically at 492 nm. In this ELISA, an increase of IgA1 protease-induced substrate cleavage results in decreased absorbance.

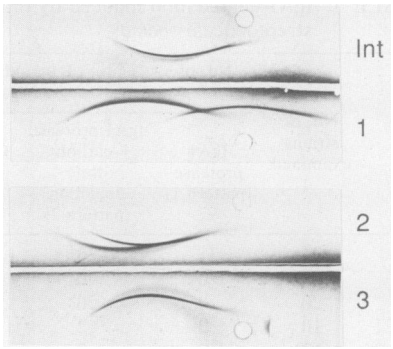

FIG. 1. IEP patterns representing three different types of changes induced in IgA1 by incubation with individual streptococcal strains. Antiserum: rabbit anti S-IgA. Patterns 1 to 3 were obtained by incubation of myeloma IgA1 (Fri) with the following representative strains: 1, *S. sanguis* ATCC 10556; 2, *S. oralis* ATCC 10557; 3, *S. mitis* biovar 2 strain SK96. Int, Intact IgA1 (Fri) control.

RESULTS

Bacterium-induced IgA1 degradation. Each of the 143 streptococcal strains representing the seven species *S. sanguis*, *S. gordonii*, *S. oralis*, *S. mitis*, *S. salivarius*, *S. anginosus*, and *S. mutans* were incubated with human IgA1. Examination of the reaction product by IEP revealed that 62 of the 143 strains induced change in the substrate. Three types of changes, each represented by a characteristic IEP pattern, were observed (Fig. 1). Patterns 1 and 2 were characterized by the presence of two separate precipitation lines which, by their reactivity with antisera specific for light chains, α chains, and Fc_α, were identified as Fab and Fc as described previously (16). Accordingly, strains inducing these patterns were identified as IgA1 protease producers. The general finding of a pattern consisting of two lines showing a reaction of immunological unrelatedness indicated that total cleavage of the substrate into Fab and Fc had taken place. Whereas cleavage pattern 1 was characterized by Fab and Fc fragments having different electrophoretic mobilities, the two fragments of pattern 2 showed similar mobilities (Fig. 1). This difference was due to a different mobility of Fc rather than of Fab fragments and was more pronounced with some myeloma IgA1 substrates than with others. In the third

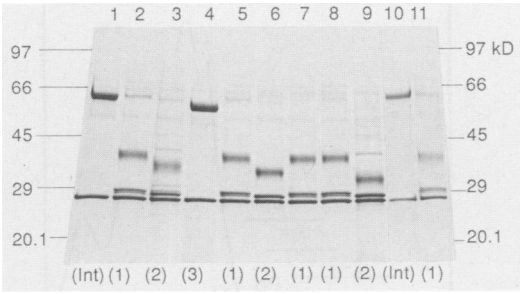

FIG. 2. SDS-PAGE analysis of IgA1 (Fri) substrates incubated with strains of streptococci. The degradation pattern obtained by IEP is indicated for each substrate below each lane. Lanes: 1, IgA1 (Fri) control; 2 to 8, effect of individual strains representing different taxa: 2, *S. sanguis* biovar 1 ATCC 10556; 3, *S. oralis* ATCC 10557; 4, *S. mitis* biovar 2 strain SK96; 5, *S. sanguis* biovar 4 strain SK46; 6, *S. mitis* biovar 1 strain SK136; 7, *S. sanguis* biovar 2 strain SK156; 8, *S. sanguis* biovar 3 strain SK163; 9, *S. sanguis* biovar 1 ATCC 10556 in combination with *S. mitis* biovar 2 strain SK96; 10 and 11, *S. mitis* biovar 2 strain SK96 and *S. oralis* ATCC 10557, respectively, in the presence of neuraminidase inhibitor. Int, Intact IgA1 (Fri) control. kD, Kilodaltons.

type of induced change, only one precipitation line was observed, which, however, had moved closer to the cathode than intact IgA1 substrate (Fig. 1).

The three distinct patterns of degradation detectable by IEP were also detectable by SDS-PAGE analysis of IgA1 incubated with representative strains (Fig. 2). Whereas light chains were invariably left intact, all strains representing degradation patterns 1 and 2, i.e., IgA1 protease producers, gave rise to bands of α-chain fragments which, by their apparent M_r, were identified as Fc and Fd. The Fc produced from IgA1 (Fri) by strains representing pattern 1 demonstrated an M_r of 40,000, whereas a lower M_r of 36,000 was calculated for Fc of pattern 2 (Fig. 2). A similar difference of the Fc fragments characteristic of pattern 1 and pattern 2 was observed when IgA1 (Mor) or IgA1 (Kah) was used as the substrate. In contrast to the Fc fragments, Fd fragments of the two patterns appeared to be of similar molecular weights. However, when IgA1 (Fri) or IgA1 (Kah) was used as the substrate, a minor strain-dependent variation was observed. With these substrates, Fd produced by pattern 1 strains travelled as a closely spaced double band (M_r ca. 31,000) whereas Fd of pattern 2 was electrophoretically homogeneous, travelling like the lower-M_r component of the pattern 1 double band (Fig. 2). The change in the IgA1 substrate observed as IEP pattern 3 was expressed in SDS-PAGE exclusively through a decrease in the molecular size of the α chain (Fig. 2).

Identification of cleavage site. The peptide bond susceptible to the IgA1 protease of *S. sanguis* ATCC 10556 has been previously identified as the Pro-Thr bond between residues 227 and 228 in the hinge region of the α1 chain (31). In this study, strain ATCC 10556 gave rise to IEP/SDS-PAGE pattern 1. Pattern 2 was characterized by an Fc of lower M_r. In searching for an explanation of this difference, the specificity of the IgA1 protease of a strain representing pattern 2 (*S. oralis* ATCC 10557) was examined. Partial N-terminal amino acid sequence analysis of Fc fragments resulting from IgA1 cleavage with this enzyme revealed the following: Thr-Pro-(—)-Pro-(—)-Thr-Pro-Pro-Thr-Pro-(—)-Pro-. Unidentified residues, indicated by dashes, probably correspond to glycosylated serine residues. When aligned with the primary structure of the α1 chain (35), the sequence indicates that the IgA1 protease of *S. oralis* ATCC 10557 cleaves the Pro-Thr peptide bond at position 227-228, in analogy with the *S. sanguis* ATCC 10556 IgA1 protease.

Carbohydrate depletion of IgA1. In search of an alternative explanation of the observed electrophoretic patterns, the effect of representative strains on the carbohydrate moiety of the IgA1 substrate was examined. Carbohydrate analysis of IgA1 exposed to streptococcal strains representing the three IEP/SDS-PAGE patterns revealed that the strain giving rise to pattern 1 (*S. sanguis* ATCC 10556) left the carbohydrate moiety intact. In contrast, the strains giving rise to patterns 2 (*S. oralis* ATCC 10557) and 3 (*S. mitis* biovar 2 SK96) removed significant amounts of sialic acid, galactose, and, in the case of SK96, *N*-acetylglucosamine (Table 2).

Four experiments were performed to confirm that carbohydrate depletion of the IgA1 molecule can explain the differences observed in electrophoretic patterns. (i) Treatment of IgA1 substrate with *V. cholerae* neuraminidase before or after incubation with *S. sanguis* ATCC 10556 (pattern 1) resulted in IEP pattern 2 (not shown). (ii) Incubation of IgA1 with *S. oralis* ATCC 10557 crude enzymes (pattern 2) in the presence of neuraminidase inhibitor resulted in degradation pattern 1, as revealed by both IEP (not shown) and SDS-PAGE (Fig. 2). (iii) Coincubation of IgA1 with *S. sanguis* ATCC 10556 (pattern 1) and *S. mitis* biovar 2 SK96 (pattern 3) resulted in degradation pattern 2, as revealed by IEP (not shown). However, the SDS-PAGE examination revealed an Fc with an apparent molecular size smaller than that of the Fc obtained with *S. oralis* (Fig. 2).

TABLE 2. Quantification of carbohydrate in IgA1 (Fri) exposed to individual streptococcal strains representing three different patterns of degradation or treated with preparations of glycosidases

Bacterial strain or enzyme preparation	Degradation pattern	Mean carbohydrate content[a] (g/100 g of IgA1 protein)					
		Fucose	Mannose	Galactose	*N*-Acetyl-glucosamine	*N*-Acetyl-galactosamine	NeuNAc
Unexposed IgA1 (control)		0.20	1.36	1.66	2.27	0.96	1.33
S. sanguis ATCC 10556	1	0.19	1.22	1.62	2.31	0.89	1.32
S. oralis ATCC 10557	2	0.20	1.24	0.19	2.04	0.88	0.23
S. mitis biovar 2 strain SK96	3	0.23	1.16	0.40	1.19	0.80	0.00
Neuraminidase		0.18	1.36	1.68	2.19	0.90	0.09
S. mitis biovar 2 strain SK96 (crude enzyme)		0.21	1.22	0.36	1.23	0.88	0.02
Neuraminidase, endo-α-*N*-acetylgalactosaminidase		0.04	0.40	0.36	0.84	0.13	0.03

[a] Mean of two determinations.

TABLE 3. Antibody-induced inhibition of streptococcal IgA1 protease

IgA1 protease	No. of strains examined	Inhibition by antiserum to IgA1 protease from:				
		S. sanguis biovar				*S. oralis*[a] (SK10)
		1 (SK1)	2 (SK115)	3 (SK150)	4 (SK45)	
S. sanguis biovars 1–3	27	+	+	+	+	−
S. sanguis biovar 4	4	−	−	−	+	−
S. oralis	20	−	−	−	−	+
S. mitis biovar 1	2	−	−	−	−	−

[a] Serum from patient with subacute endocarditis due to *S. oralis*.

This difference correlated with the ability of *S. mitis* biovar 2 SK96, but not *S. oralis*, to release *N*-acetylglucosamine from IgA1 (Table 2). (iv) Incubation of IgA1 with *S. mitis* biovar 2 SK96 crude enzyme (pattern 3) in the presence of neuraminidase inhibitor did not cause any change in the substrate as revealed by both IEP (not shown) and SDS-PAGE (Fig. 2).

Correlation between IgA1 degradation patterns and identity of strains. The correlation between the identity of the streptococcal strains and the IgA1 degradation pattern induced by them is shown in Table 1. As shown, IgA1 protease-induced changes were characteristic of all strains of *S. sanguis* and *S. oralis* examined and of 2 of 10 strains of *S. mitis* biovar 1. Strains of *S. oralis* and the two IgA1 protease-producing *S. mitis* biovar 1, in addition, caused degradation of the carbohydrate moiety of the IgA1 substrate (pattern 2). Finally, 8 of the 13 strains of *S. mitis* biovar 2 and 1 strain of *S. anginosus* (ATCC 27335) attacked only the carbohydrates of the substrate (pattern 3). No other strain of *S. gordonii*, *S. salivarius*, *S. anginosus*, or *S. mutans* was capable of degrading IgA1.

Antibody-induced inhibition of IgA1 proteases. Rabbits were immunized with protease preparations produced from five streptococcal strains representing each of the four biovars of *S. sanguis* (SK1, SK115, SK150, and SK45) and *S. oralis* (SK10). Rabbits immunized with *S. sanguis* IgA1 proteases responded after an immunization period of 1 year with titers of neutralizing antibodies from 8 to 256. Purified immunoglobulins of sera from rabbits immunized with *S. sanguis* biovar 1, 2 and 3 strains caused complete inhibition of IgA1 proteases from any strain belonging to these three biovars but were unable to inhibit IgA1 proteases of *S. sanguis* biovar 4 strains. In contrast, antibodies raised against the *S. sanguis* biovar 4 strain inhibited IgA1 proteases of strains of all four biovars of *S. sanguis* (Table 3). None of the antibody preparations against *S. sanguis* proteases affected the activity of IgA1 proteases from *S. oralis* and *S. mitis*.

None of four rabbits, two rats, and one guinea pig injected with different preparations of IgA1 protease prepared from *S. oralis* SK10 responded with detectable protease-neutralizing antibodies, in some cases even after repeated injections over a period of more than 2 years. However, a serum obtained from a patient with subacute endocarditis due to *S. oralis* was able to inhibit (titer, 1,024) IgA1 proteases from all 20 strains of *S. oralis* examined, but had no effect on proteases from *S. sanguis* and *S. mitis* (Table 3).

Purification of IgA1 proteases. The protein profile obtained by size-exclusion chromatography of a crude enzyme preparation from *S. oralis* ATCC 10557 is presented in Fig. 3. Maximal IgA1 protease activity was eluted with an elution volume corresponding to an apparent M_r of 100,000. A preparation of IgA1 protease devoid of β-galactosidase but containing neuraminidase activity was isolated by pooling eluent fractions as indicated on the figure. By analogous fractionation of crude enzyme from *S. sanguis* ATCC 10556, the IgA1 protease of this strain was found to elute at a position similar to that of the *S. oralis* protease (not shown). In this case neuraminidase and β-galactosidase were not detected, and a pool of protease-active eluent fractions were isolated as the source of purified IgA1 protease.

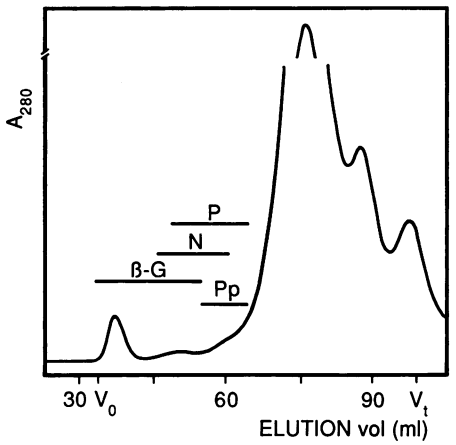

FIG. 3. Size-exclusion chromatography of crude extracellular enzymes from *S. oralis* ATCC 10557 on a column of Superose 12. Proteins were eluted with 0.1 M phosphate buffer, pH 7.0, at 1 ml/min. Elution positions for IgA1 protease (P), neuraminidase (N), and β-galactosidase (β-G) are indicated by horizontal bars. A pool of eluted fractions (P_p) was isolated as the source of purified IgA1 protease. V_0, void volume; V_t, total column volume; A_{280}, optical density at 280 nm.

Effect of glycosidase treatment on IgA1 and its susceptibility to cleavage by IgA1 proteases. Carbohydrate analysis of glycosidase-treated IgA1 (Fri) substrate (Table 2) indicated that neuraminidase from *V. cholerae* had caused specific and almost complete removal of sialic acid. Treatment with crude enzymes from *S. mitis* biovar 2 SK96 had removed sialic acid, galactose, and a significant proportion of *N*-acetylglucosamine, in analogy with what was observed after treatment with *S. mitis* biovar 2 SK96 whole cells (Table 2). As expected, treatment with neuraminidase and endo-α-*N*-acetylgalactosaminidase had removed sialic acid, galactose, and *N*-acetylgalactosamine. However, significant amounts of fucose, mannose, and *N*-acetylglucosamine had also been removed (Table 2), probably due to heterogeneity of the commercial preparation of endo-α-*N*-acetylgalactosaminidase. Progress curves for *S. sanguis* ATCC 10556 IgA1 protease-induced cleavage of the three glycosidase-treated IgA1 (Fri) substrates are presented in Fig. 4A. Because the progress curves obtained for cleavage of the three corresponding sham-treated control substrates were almost congruent, only one control curve is included in the figure. The observed steeper decline of ELISA readings for neuraminidase-treated than for control substrate indicates that removal of terminal sialic acid from the carbohydrate side chains of the substrate resulted in significantly more cleavage during the following incubation with IgA protease. The same increase of cleavage by protease was observed after treatment of IgA1 with crude extracellular enzymes

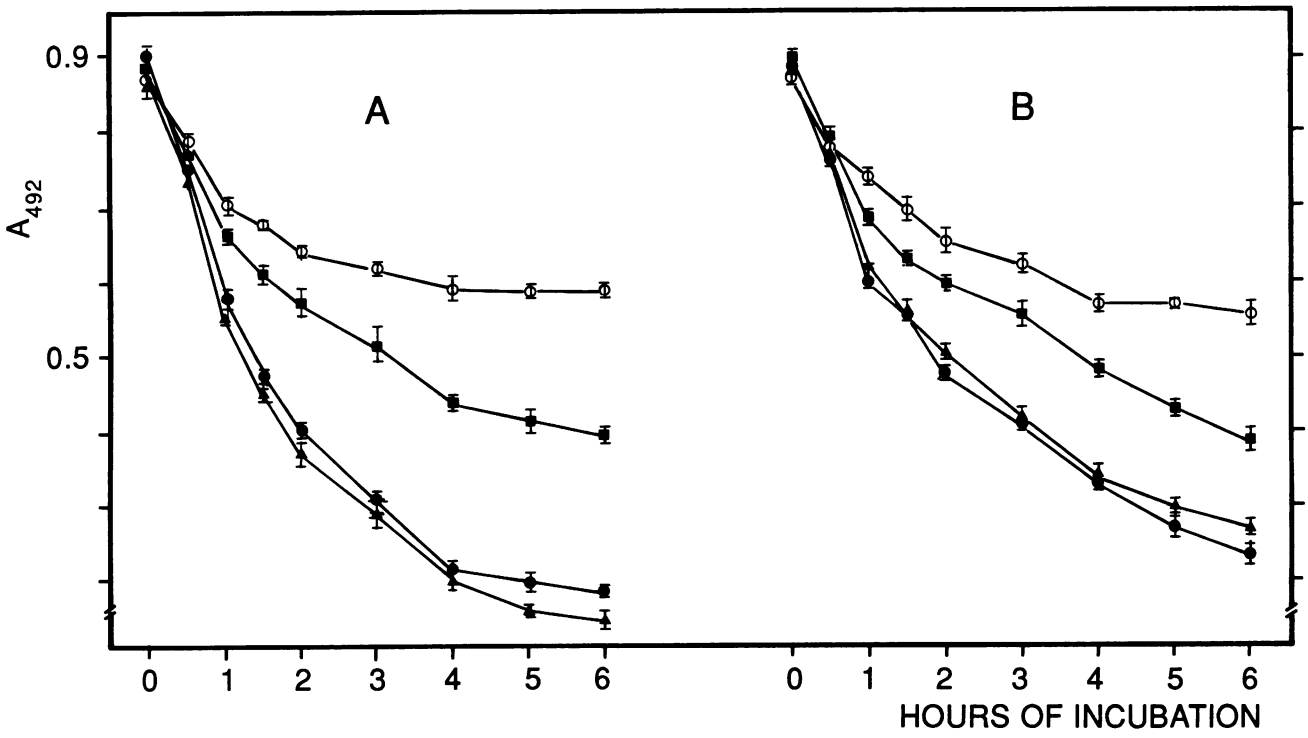

FIG. 4. ELISA curves illustrating the effect of three different glycosidase treatments on the susceptibility of IgA1 (Fri) to cleavage during subsequent incubation with IgA1 protease from *S. sanguis* ATCC 10556 (A) or *S. oralis* ATCC 10557 (B). ELISA readings (A_{492}; error bars show mean ± SE of triplicate determinations), which reflect the proportion of IgA1 substrate molecules still carrying Fab, are plotted against incubation time. The four curves represent untreated control IgA1 (■) and the three IgA1 substrates treated with *V. cholerae* neuraminidase (▲), crude enzymes from *S. mitis* biovar 2 strain SK96 (●), or *V. cholerae* neuraminidase in combination with endo-α-*N*-acetylgalactosaminidase (○).

from *S. mitis* biovar 2 SK96. In contrast, IgA1 treated with neuraminidase and endo-α-*N*-acetylgalactosaminidase was found to be cleaved to a lesser extent than sham-treated control substrate (Fig. 4A). The possibility that the latter finding was due to degradation of IgA1 protease by proteases contaminating the glycosidase preparations used for pretreatment of the substrate could be excluded. Thus, in a control experiment, the activity of *S. sanguis* IgA protease was found to be the same whether it was solubilized in PBS or in a solution of neuraminidase and endo-α-*N*-acetylgalactosaminidase having undergone the same incubation, dialysis, and dilution procedures as used in the treatment of IgA1 substrate.

The same substrates were tested for susceptibility to IgA1 protease from *S. oralis* ATCC 10557. The pattern of the progress curves obtained (Fig. 4B) paralleled that obtained by use of the protease from *S. sanguis* ATCC 10556 (Fig. 4A).

Following the same protocol, two other IgA1 myeloma proteins (Kah and Mor) were subjected to the same glycosidase treatments and tested for altered susceptibility to IgA1 protease from *S. sanguis* ATCC 10556. With both of these substrates, the effect of the individual glycosidase treatments on susceptibility to IgA1 protease was qualitatively similar to the effect observed when IgA1 (Fri) was employed (not shown). However, with these substrates, the increase of susceptibility to IgA1 protease activity caused by treatment with neuraminidase or *S. mitis* biovar 2 SK96 crude enzyme was less than that observed with IgA1 (Fri).

DISCUSSION

This study demonstrated that all strains of *S. sanguis* (biovars 1 to 4) and *S. oralis* (previously *S. mitior*/*S. sanguis* II) examined produced IgA1 protease. In addition, 2 of 10 strains of *S. mitis* biovar 1 showed IgA1 protease activity. Previously published observations that only some strains of the two species *S. sanguis* and "*S. mitior*" possess IgA1 protease activity (8, 15) can be explained by a previous unsatisfactory taxonomy of this group of bacteria. A recent taxonomic study (18), which employed the strain collection used in the present study, demonstrated that IgA1 protease-negative strains previously classified as *S. sanguis* belong to a separate species, *S. gordonii* sp. nov. Likewise, protease-negative strains previously assigned to *S. oralis* ("*S. mitior*") belong in the species *S. mitis* (biovar 1).

Our partial amino acid sequence analysis of the N-terminus of Fc fragments of IgA1 cleaved by the *S. oralis* IgA1 protease revealed that this enzyme attacks the same peptide bond as that cleaved by the IgA1 protease from *S. sanguis* (31) and *S. pneumoniae* (16), i.e., the Pro-Thr bond at position 227-228 in the IgA1 hinge region. Although amino acid analysis of the cleavage site induced by the *S. mitis* protease was not performed, the identical size of the resulting Fd fragment as revealed by SDS-PAGE analysis (Fig. 2) indicates the same specificity for this enzyme.

Streptococcal IgA1 proteases thus appear to share a common specificity, which would suggest a common enzyme structure and a common phylogeny. Nevertheless, Gilbert et

al. (11) recently demonstrated that a cloned IgA1 protease gene from *S. sanguis* fails to hybridize with DNA from protease-producing *S. pneumoniae*. This may reflect the distant genetic relationship (20 to 30% DNA-DNA homology) previously observed between these two species (22). A closer relationship has been observed between *S. pneumoniae*, *S. oralis*, and *S. mitis* based on genomic DNA-DNA hybridization experiments (22). Whether this is reflected in a closer relationship of the *iga* gene of these three species is not yet known. In the present context, however, it is interesting that these three species, in addition to the IgA1 protease activity, shared glycosidase activity capable of removing significant parts of the carbohydrate moiety of the IgA1 molecule (Tables 1 and 2) (16).

A total of four distinct IgA1 proteases were detected among the streptococcal strains examined, as revealed by examination with enzyme-neutralizing antibodies: one shared by all strains belonging to *S. sanguis* biovars 1, 2 and 3, one in strains of *S. sanguis* biovar 4, which have been shown to differ from other *S. sanguis* strains also by cell wall antigens (18), one shared by all strains of *S. oralis*, and one or possibly more types in strains of *S. mitis* (Table 3). The lack of diversity of IgA1 proteases in the species *S. sanguis* and *S. oralis* demonstrated here is surprising in view of our previous demonstration of at least 15 different antigenic types of IgA1 proteases in *H. influenzae* (21). As both *S. sanguis* and *S. oralis* remain constant and numerically significant members of the human oral flora throughout life, this may indicate that IgA1 proteases of the oral streptococci are not exposed to a significant selection pressure by neutralizing antibodies in vivo. This conclusion is supported by the observation by Gilbert et al. (12) that pooled human colostrum contains very low titers of neutralizing antibodies to *S. sanguis* IgA1 protease in contrast to other bacterial IgA1 proteases.

In spite of the identical specificities of their IgA1 proteases, *S. sanguis*, *S. oralis*, and protease-producing strains of *S. mitis* biovar 1 induced clearly distinct IgA1 degradation patterns as revealed by both IEP (Fig. 1) and SDS-PAGE (Fig. 2) analyses. A series of experiments, which included inhibition of neuraminidase activity and quantitative carbohydrate analyses, demonstrated that removal of carbohydrate was responsible for these different patterns. Among the remaining streptococcal taxa, *S. mitis* biovar 2 was unique in attacking the carbohydrate moiety of IgA1 without having IgA1 protease activity (Table 2). The neuraminidase from a strain designated *S. sanguis* (presumably *S. oralis*) has previously been isolated and characterized (49). It is distinct from the neuraminidase of *V. cholerae* and *Clostridium* species in being able to release O-substituted sialic acids from glycoconjugates. Our finding that blockage of the *S. oralis* or *S. mitis* neuraminidase prevented other relevant glycosidases from attacking the carbohydrate moiety of IgA1 suggests that the latter enzymes (i.e., β-galactosidase, *N*-acetylglucosaminidase) are exoglycosidases, which require initial removal of terminal sialic acid for their activity.

The α1 heavy-chain of IgA1 molecules carries several carbohydrate side chains of different composition. In the Fc, they are of the complex, biantennary type terminating in either NeuNAc(α2-6)Gal(β1-4)GlcNac(β1-2)Man- or Gal (β1-4)GlcNAc(β1-2)Man- (1). Oligosaccharides of this type may be present also in the variable domain (48). In the hinge region, the α1 chain carries five small oligosaccharides O-linked to serine residues. By analysis of a myeloma protein, Baenziger and Kornfeld (2) found that four of these consisted of Gal(β1-3)GalNAc-, whereas one contained a single residue of GalNAc only. However, experiments with other IgA1 myeloma proteins (33, 44) and polyclonal serum IgA1 (A. Pierce-Cretel, thesis no. 595, Université des Sciences et Techniques, Lille, France, 1983) have demonstrated that the four Gal(β1-3)GalNAc- side chains may have terminal sialic acid. This was also the case for the IgA1 (Fri) employed in the present study.

Considering the distribution and structure of IgA1 oligosaccharides and the results shown in Table 2, it can be inferred that *S. oralis* and some strains of *S. mitis* biovars 1 and 2, in addition to removing sialic acid, caused extensive degradation of the remaining part of the oligosaccharides both within and outside the hinge region. Similar activity has previously been demonstrated in *S. pneumoniae* (16). Furthermore, changes in the electrophoretic mobility of IgA1 indicative of carbohydrate degradation have been observed in strains of several oral bacteria, including *Actinomyces viscosus* and *Actinomyces naeslundii* (7).

These results raise the interesting question of whether bacteria can interfere with the functions of IgA or other immunoglobulins by attacking the carbohydrate moiety. However, the significance of carbohydrates per se for the function of IgA molecules is not known. Previous, but unconfirmed, observations have indicated that removal of carbohydrate from IgA2 allotype A2m(1), which lacks covalent binding between heavy and light chains, destroys the structural integrity of the antibody molecule (5). Other studies have demonstrated that carbohydrate-depleted mouse monoclonal IgG fails to activate complement and bind to phagocytes (29). The possible significance of the carbohydrate moiety of IgA should be seen in light of the extensive heterogeneity in glycosylation of S-IgA proteins (43), different IgA myeloma proteins (45), and even within single IgA myeloma proteins. The latter is evident from our observation that a size heterogeneity of Fd fragments of a myeloma protein was eliminated by incubation with glycosidases (Fig. 2). However, the carbohydrate moiety of S-IgA molecules is to a large extent responsible for the hydrophilicity which is unique to this isotype, and forms a basis for some of its important functions. Thus, it is conceivable that bacteria, by removing carbohydrate from IgA, may escape some of its antibacterial functions.

Although the significance of the carbohydrate moiety of IgA antibodies per se remains to be demonstrated, the present experiments show that depletion of carbohydrate may change the susceptibility of IgA1 molecules to IgA1 protease activity. Several lines of evidence have indicated that IgA1 proteases recognize only a small segment of the α1 chain within the hinge region (32). Kornfeld and Plaut (23) considered the possibility that carbohydrates in the hinge region contribute to the exquisite specificity of IgA1 proteases but noted that removal of sialic acid by neuraminidase treatment did not prevent cleavage of IgA1. Our experiments support and extend this observation by showing that cleavage of IgA1 by streptococcal IgA1 proteases is dependent on neither sialic acid nor galactose as the terminal sugar. Furthermore, these experiments indicate that sialic acid contributes to the resistance of IgA1 to protease, in analogy to what has been observed in other sialo-glycoprotein-protease systems (40).

The possibility of cooperation between IgA1 protease and a glycosidase of streptococci has been previously suggested by Labib et al. (24) on the basis of the observation that the IgA1 protease of *S. sanguis* ATCC 10556 copurified with invertase from this strain. However, cooperation between these enzymes is unlikely, particularly in view of our obser-

vation that *S. sanguis* ATCC 10556 did not affect the carbohydrate moiety of IgA1 (Table 2).

IgA1 substrates treated with neuraminidase and a commercial preparation of endo-α-*N*-acetylgalactosaminidase were found to be more resistant to IgA1 protease activity than their untreated counterparts. Judged by the effect on the content of *N*-acetylgalactosamine (Table 2), this treatment completely removed more than 85% of the hinge region oligosaccharides. Hence, our result indicate that the presence of proximal *N*-acetylgalactosamine residues is of importance for the interaction of streptococcal IgA1 proteases with the α1 chain. This interpretation is in accordance with the observation by Burton et al. (4) that aglycosyl peptide analogs of the hinge region, which are competitive inhibitors of the *N. gonorrhoeae* IgA1 proteases, do not inhibit the protease from *S. sanguis*. The possibility that *N*-acetylgalactosamine residues have a receptor function for IgA1 protease is, however, contradicted by the observation by Kornfeld and Plaut (23) as well as ourselves (unpublished) that cleavage of IgA1 by *S. sanguis* IgA1 protease is unaffected by the presence of 50 mM *N*-acetylgalactosamine. Alternatively, it may be speculated that glycosylation confers on the hinge region a conformation suitable for interaction with the protease. The relevance of this possibility is supported by the recent observation that the equilibrium conformation of a tetrapeptide analog of the IgA1 hinge region spanning the Pro-Thr bond susceptible to streptococcal IgA1 proteases is destabilized by acetylation (41). Like the hinge region oligosaccharides, carbohydrates located in Fc and Fab can be expected to influence the overall conformation of the IgA1 molecule and, possibly, its susceptibility to IgA1 protease. Whether depletion of carbohydrates in Fc or Fab contributed to the changes in susceptibility observed in these experiments is unknown.

The ability of oral streptococci to initiate plaque formation has been explained by their ability to interact with specific receptors in the acquired pellicle covering dental enamel (10, 30, 42). Our recent finding that the vast majority (87.5%, median) of the streptococci that colonize dental enamel within the first 4 h in vivo produce IgA1 protease, as opposed to corresponding proportions of only 2 and 10% on the dorsum of the tongue and the mucosa of the oropharynx, respectively (20), may indicate that evasion of the adherence-inhibiting effect of S-IgA is another important factor in this process. This possibility is further supported by the observation that the capacity of S-IgA to inhibit the adherence of oral streptococci to saliva-coated hydroxyapatite in vitro is reduced by IgA1 protease (38).

A considerable amount of data have indicated that lectinlike interactions, involving bacterial surface components and immobilized or free salivary glycoproteins, play an important role in the colonization of tooth surfaces as well as in the clearance of bacteria by agglutination in saliva. Considering that sialic acid and galactose residues have been found to be involved as receptor structures in such interactions (6, 9, 25) it is likely that the neuraminidase and galactosidase activity of oral streptococci may interfere with some of the colonization and agglutination phenomena mediated by lectinlike interactions. The overall effect of this glycosidase activity on plaque formation is still unknown. However, the present experiments suggest that the neuraminidase activity of oral streptococci belonging to *S. oralis* or *S. mitis* may promote the formation of plaque indirectly by enhancing the degradation of S-IgA1 antibodies by streptococcal IgA1 protease. The observation that neuraminidase treatment increases the susceptibility of IgA1 not only to the IgA1 protease of a neuraminidase-producing streptococcus (*S. oralis* ATCC 10557) but also to the protease produced by a strain of *S. sanguis* adds to the potential effect of this mechanism. The interesting question of whether neuraminidase released by oral streptococci may also increase the susceptibility of S-IgA1 to IgA1 proteases produced by the suspected periodontium-pathogenic *Bacteroides* and *Capnocytophaga* species is left for future studies to examine.

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