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Inhibition of *Prevotella* and *Capnocytophaga* Immunoglobulin A1 Proteases by Human Serum

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Oral *Prevotella* and *Capnocytophaga* species, regularly isolated from periodontal pockets and associated with extraoral infections, secrete specific immunoglobulin A1 (IgA1) proteases cleaving human IgA1 in the hinge region into intact Fab and Fc fragments. To investigate whether these enzymes are subject to inhibition in vivo in humans, we tested 34 sera from periodontally diseased and healthy individuals in an enzyme-linked immunosorbent assay for the presence and titers of inhibition of seven *Prevotella* and *Capnocytophaga* proteases. All or nearly all of the sera inhibited the IgA1 protease activity of *Prevotella buccae*, *Prevotella oris*, and *Prevotella loescheii*. A minor proportion of the sera inhibited *Prevotella buccalis*, *Prevotella denticola*, and *Prevotella melaninogenica* IgA1 proteases, while no sera inhibited *Capnocytophaga ochracea* IgA1 protease. All inhibition titers were low, ranging from 5 to 55, with titer being defined as the reciprocal of the dilution of serum causing 50% inhibition of one defined unit of protease activity. No correlation between periodontal disease status and the presence, absence, or titer of inhibition was observed. The nature of the low titers of inhibition in all sera of the IgA1 proteases of *P. buccae*, *P. oris*, and *P. loescheii* was further examined. In size exclusion chromatography, inhibitory activity corresponded to the peak volume of IgA. Additional inhibition of the *P. oris* IgA1 protease was found in fractions containing both IgA and IgG. Purification of the IgG fractions of five sera by passage of the sera on a protein G column resulted in recovery of inhibitory IgG antibodies against all three IgA1 proteases, with the highest titer being for the *P. oris* enzyme. These findings indicate that inhibitory activity is associated with enzyme-neutralizing antibodies.

Destructive periodontal diseases are believed to be a result of disturbances in an otherwise harmonious relationship between the oral resident microflora and the host (29). Of particular interest are disturbances brought about by microbial interference with the functions of the immune system and tissue reactions. One example of such interference is the specific immunoglobulin A1 (IgA1) proteases produced by oral *Prevotella* and *Capnocytophaga* species, which inhabit periodontal pockets (5, 12, 30–33, 40, 42) and may be isolated from extraoral infections (7, 36).

Apart from those produced by the oral *Prevotella* and *Capnocytophaga* species and the streptococcal species that initiate dental plaque formation (9, 18, 35), specific IgA1 proteases are produced by a number of pathogenic species causing diseases that take place at or originate from mucosal surfaces, where IgA1 is the prime mediator of specific immunity. One striking example of such organisms is the three leading causes of bacterial meningitis, *Haemophilus influenzae*, *Neisseria meningitidis*, and *Streptococcus pneumoniae* (20, 26, 37). Closely related, nonpathogenic *Haemophilus* and *Neisseria* species lack IgA1 proteases.

The presence of in vivo activity of IgA1 proteases from *Prevotella* and *Capnocytophaga* species has been demonstrated indirectly and in a significantly higher proportion of sera from adults with periodontal disease compared to sera from control individuals (10, 11). IgA1 proteases of these species are not inhibited by physiological protease inhibitors such as α_2-macroglobulin and α_1-proteinase inhibitor (9, 13, 34). Subcutaneous injection of IgA1 protease preparations from *Prevotella* and *Capnocytophaga* in rabbits induce enzyme-neutralizing antibodies. However, it is not known if colonization or infection gives rise to production of inhibitory antibodies in humans. Enzyme-neutralizing antibodies against IgA1 proteases of overt pathogens as well as of oral streptococci involved in extraoral infections have been observed (3, 4, 14, 15, 25, 39).

We have previously demonstrated that individual *Prevotella* and *Capnocytophaga* species produce antigenically distinct IgA1 proteases, although three pairs of species produce proteases sharing antigenic determinants, i.e., *Prevotella oralis* and *Prevotella veroralis*, *Capnocytophaga ochracea* and *Capnocytophaga sputigena*, and *Capnocytophaga gingivalis* and *Capnocytophaga granulosa* (9, 13).

The purpose of the present study was to investigate the ability of sera from healthy individuals and periodontally diseased, but otherwise healthy, individuals to inhibit IgA1 proteases from *Prevotella* and *Capnocytophaga* species and to determine the nature of the inhibitory activity.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The following strains were included in the study: *Prevotella melaninogenica* ATCC 25845^T (American Type Culture Collection, Rockville, Md.), *Prevotella buccae* CCUG 93811 (ATCC 33574^T) (Culture Collection of the University of Göteborg, Göteborg, Sweden), *Prevotella buccalis* CCUG 15557 (NCDO 2354^T) (National Collection of Dairy Organisms, Reading, United Kingdom), *Prevotella oris* CCUG 15405 (ATCC 33573^T), *Prevotella denticola* CCUG 15558 (NCDO 2352^T), *Prevotella loescheii* CCUG 5914 (ATCC 15930^T), and *C. ochracea* CCUG 9716 (ATCC 27872^T). All strains had detectable IgA1 protease activity. The strains were cultivated on plaque agar (17).

IgA1 preparations. Human dimeric IgA1 (Kah) carrying λ-light chains was isolated from the serum of a patient with multiple myelomas as previously described (8). For one experiment monomeric IgA1 (Mor) was obtained by separation of the IgA1 Mor preparation by size exclusion chromatography (2).

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IgA1 protease preparation and calibration. Crude IgA1 protease preparations were prepared from all bacterial strains by the method of Higerd et al. (16) with plaque agar as the propagation medium and with the final protease preparation in 0.01 M phosphate-buffered saline (PBS) (2 mM NaH_2PO_4–H_2O, 8 mM Na_2PO_4–$2H_2O$ [pH 7.4], 154 mM NaCl, 0.2 mg of NaN_3 per ml). The protease preparations were aliquoted, and *Prevotella* proteases were stored at −60°C while the *C. ochracea* protease was kept at 4°C.

Antisera were raised in rabbits against proteases from the above-mentioned strains as previously described (9).

For determination of activity, each IgA1 protease preparation was serially twofold diluted in PBS containing 1 mg of bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.) per ml (PBS-BSA). Triplicate samples (25 μl) of diluted protease were each incubated with 25 μl of IgA1 (Kah) at a concentration of 0.4 mg/ml for 18 h at 37°C. IgA1 incubated with PBS-BSA instead of protease served as a control. The proportion of IgA1 cleaved in each well was determined in an enzyme-linked immunosorbent assay (ELISA) as described by Reinholdt (38). After incubation, 275 μl of PBS-BSA was added to all wells. A volume of 100 μl was withdrawn from each well and transferred to a microtiter plate (Nunc, Roskilde, Denmark) with a catching layer of mouse monoclonal antibody specific for Fc of human IgA1 (code MAHu/IgA1/MAb, 70 ng/ml; Nordic, Tilburg, The Netherlands) immobilized via a coating layer of rabbit antibodies specific for mouse Igs (code Z0259, 1:2,000; Dako, Glostrup, Denmark). The assay measured residual intact IgA1 by development with peroxidase-conjugated rabbit anti-human λ-light chains (code P0130, 1:1,000; Dako). Titration curves were drawn with the Fig P program (Biosoft, Cambridge, United Kingdom) with a regression function (first-order monoexponential decay with residual). One unit of IgA1 protease activity was defined as the dilution (prior to mixing with the substrate) causing 75% cleavage of the IgA1 (Kah) substrate under the conditions used in the assay, as reflected by the optical density (OD) obtained for the mixture of protease and IgA1 relative to the OD obtained for the IgA1 incubated with buffer.

Serum samples. Sera were prepared from blood samples obtained from 34 individuals, including 7 with juvenile periodontitis (17 to 22 years of age), 5 juvenile controls (19 to 21 years of age), 8 with rapidly progressive periodontitis (23 to 36 years of age), 7 with adult periodontitis (37 to 65 years of age), and 2 adult controls (29 to 52 years of age). Specific clinical criteria were as described previously (24). Serum samples were numbered consecutively, aliquoted, and stored at −60°C.

Serum from three hypogammaglobulinemic individuals was kindly donated by W. Friman (Clinic of Infectious Diseases, Östra Sjukhuset, Göteborg, Sweden).

Approximately 10 ml of serum was collected from each of five additional individuals. Serum from one of these individuals was fractionated by size exclusion chromatography on a Superose 6B column (Pharmacia, Uppsala, Sweden) equilibrated in 0.05 M PBS, pH 7.4 (10 mM NaH_2PO_4–H_2O, 40 mM Na_2PO_4–$2H_2O$, 154 mM NaCl, 0.2 mg of NaN_3 per ml). Eluted protein was detected spectrophotometrically at 280 nm. A volume of 1 ml of serum was chromatographed at a flow rate of 0.25 ml/min (fraction size, 1.5 ml). Fractions diluted 1:100 were assayed for Ig contents in an ELISA using, for detection of IgM, rabbit anti-human μ chains (1:2,000; Dako) for capture and the same antibody (peroxidase conjugated, 1:1,000; Dako) for development. For detection of IgG, rabbit anti-human γ-light chains (1:2,000; Dako) were used for capture and a mixture of peroxidase-conjugated anti-human κ- and λ-light-chain antibodies was used for development (Dako; see below). IgA-containing fractions were detected by the ELISA described below.

The IgG fraction was purified from all five sera on a 1-ml HiTrap protein G column by following the manufacturer's (Pharmacia Biotech, Uppsala, Sweden) instructions. The IgG concentrations in sera and the corresponding IgG fractions were kindly determined by U. Gerdes by standard procedures at the Department of Clinical Biochemistry, Aarhus University Hospital, Aarhus, Denmark.

Determination of serum-induced inhibition of IgA1 proteases. The assay was developed by Reinholdt (38). In principle, protease adjusted to 1 U of protease activity (see Materials and Methods) was preincubated with serially diluted serum, allowing inhibitory substances in the serum to react with the enzyme. Since IgA1 antibodies may be, at the same time, substrate for and inhibitory antibodies to the IgA1 proteases, myeloma IgA1 was added after the preincubation period to ensure the presence of the substrate for residual protease activity. An ELISA was subsequently used to measure intact IgA1 in the reaction mixture and to compare the OD to that obtained with serially diluted serum plus substrate myeloma IgA1 without protease (intact substrate control) and to that obtained with uninhibited protease (1 U of protease activity). Briefly, twofold dilutions (25 μl) of serum in PBS-BSA starting with 1:5 were added to two rows of a microtiter plate. One row received 25 μl of protease preparation adjusted to 1 U of IgA1 protease activity. This was termed the reaction mixture. The other row of diluted serum received PBS-BSA instead of protease and served as the intact substrate control. The microtiter plate was incubated for 90 min to allow the protease and inhibitory molecules to interact. Subsequently, 25 μl of myeloma IgA1 at a concentration of 20 μg/ml was added to all wells in both rows. The mixtures were further incubated for 18 h at 37°C to enable residual IgA1 protease to cleave IgA1. After incubation, 275 μl of PBS-BSA was added to each well. Cleavage of IgA1 was measured as in the ELISA described above for protease activity determination, except that the coating layer was fluorescein isothiocyanate (FITC)-conjugated $F(ab')_2$ fragments of rabbit anti-mouse Igs (code F0313; Dako) and that the developing layer was a mixture of peroxidase-conjugated anti-human κ- and λ-light-chain antibodies (codes P0129 and P0130; Dako) at saturating conditions (see Results). Triplicate 100-μl samples of the reaction mixture and duplicate samples of the intact substrate control were transferred to a microtiter plate (Nunc). One row of the microtiter plate with only the coating layer also received the intact substrate control in order to check the reaction of serum with this substrate. Curves were drawn with the Fig P program (Biosoft), and the inhibition titer was defined as the dilution of serum (prior to its being mixed with the protease) giving rise to 50% inhibition of IgA1 protease activity relative to the OD obtained with the intact substrate control and to that obtained with 1 U of protease activity (corresponding to the lower horizontal part of the reaction mixture titration curve). Calculated titers of less than 50% inhibition were recorded as 0.

TABLE 1. Mutual inhibition of peroxidase-conjugated rabbit anti-human λ-light-chain and κ-light-chain antibodies[a]

Purified Ig detected	Dilution of anti-human light-chain type:		OD (%[b])
	λ	κ	
IgA1 Kah (λ-light-chain antibodies)	1:1,000		0.802 (100)
	1:1,000	1:250	0.515 (62)
	1:1,000	1:500	0.588 (71)
	1:1,000	1:1,000	0.655 (79)
IgA1 Mor (κ-light-chain antibodies)		1:1,000	0.721 (100)
	1:250	1:1,000	0.417 (58)
	1:500	1:1,000	0.450 (62)
	1:1,000	1:1,000	0.489 (68)

[a] One hundred microliters of myeloma IgA1 Kah (λ-light chains) or myeloma IgA1 Mor (κ-light chains) (20 μg/ml) was immobilized in quintuples in microtiter wells (see Materials and Methods for the coating and catching layers) and visualized by additions of different combinations of peroxidase-conjugated anti-λ- and anti-κ-light-chain antibodies.

[b] Percentage of samples with this OD.

RESULTS

Assay for detection of serum-induced inhibition of IgA1 proteases. The assay for detection of serum-induced inhibition of IgA1 proteases had been validated with IgA1 proteases from *H. influenzae*, *N. meningitidis*, and streptococci (38). The ability of the assay to measure inhibition of *Prevotella* and *Capnocytophaga* IgA1 proteases was tested with inhibitory rabbit antibodies raised against each individual enzyme and previously verified for inhibition in an immunoelectrophoretic assay (9, 13). The proteases were also inhibited under the present test circumstances with titers ranging from 9 to 70, thereby demonstrating the ability of the assay to measure serum-induced inhibition of *Prevotella* and *Capnocytophaga* IgA1 proteases.

Titrable background responses of some sera in microtiter wells coated with rabbit anti-mouse Ig antibody were reported by Reinholdt (38) to disappear after replacement of this antibody with $F(ab')_2$ fragments. However, some sera from the present study still produced titratable background responses with both catching layers (Fig. 1). In most cases, the background response was highest with the intact antibody as the catching layer, but for three sera the background response with the $F(ab')_2$ antibody was equal to or higher than that with the intact antibody. Use of FITC-conjugated $F(ab')_2$ further reduced the background response (Fig. 1), for which reason this form of the antibody was used in the experiments.

The intact IgA1 that binds to the catching layer in the ELISA may be IgA1 of both light-chain types (the ratio of κ to λ in normal serum is 2:1) and myeloma IgA1 Kah of the λ-light-chain type. Therefore, both rabbit anti-human κ- and λ-light-chain antibodies were added in the developing layer. A slightly increased OD for the intact substrate control curve was

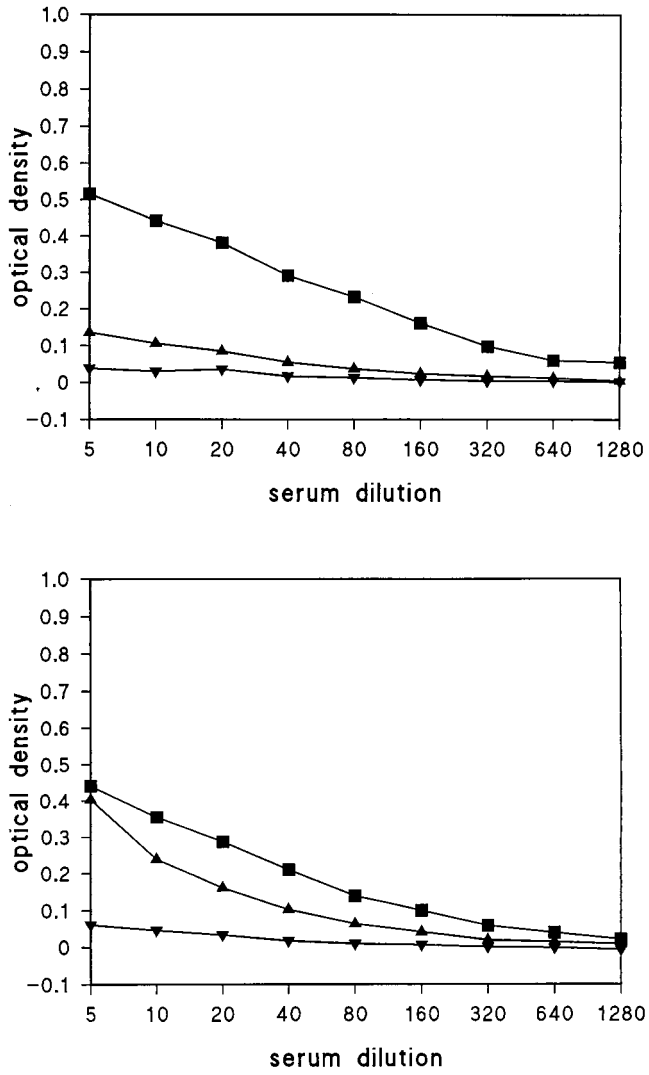

FIG. 1. Background reactions of two titrated sera in an ELISA using a coating layer of rabbit anti-mouse Ig (■), the $F(ab')_2$ of this antibody (▲), or the FITC-conjugated $F(ab')_2$ of this antibody (▼).

observed following titration of sera. In search of an explanation to this phenomenon, we discovered that each of the anti-light-chain antibodies, when kept in solution, inhibited the OD developed by the other anti-light-chain antibody bound to a myeloma IgA1 of the relevant light-chain type (Table 1). A possible explanation may be the presence of excess free λ- or κ-light chains added to the reagents to absorb cross-reactivity. The anti-λ-light-chain antibodies seemed to inhibit the anti-κ-light-chain antibodies more than the reverse. In our experiments, at high concentrations of serum, κ-light-chain antibodies preferentially bound to the capture layer, which left many unbound anti-λ-light-chain antibodies. This might explain the lower OD at the start of the intact substrate control curve. Consequently, we used anti-κ-light-chain antibodies at a dilution of 1:500 and anti-λ-light-chain antibodies at a dilution of 1:1,000, which resulted in an almost horizontal intact substrate control curve.

Inhibition of *Prevotella* and *Capnocytophaga* IgA1 proteases by human sera. Varied inhibition patterns against the six selected *Prevotella* IgA1 proteases were observed for the examined sera (Table 2). All or nearly all sera inhibited the *P. buccae*, *P. oris*, and the *P. loescheii* enzymes. Minor proportions of the sera inhibited the *P. buccalis*, *P. denticola*, and *P. melaninogenica* proteases. No sera inhibited the *C. ochracea* enzyme (data are therefore not shown in Table 2). The titers of inhibition were low in all positive sera, irrespective of the protease tested (Table 2; Fig. 2). However, the sera had a higher median titer inhibition of the *P. oris* protease than of other proteases.

In a previous investigation (11) the sera had been tested for indirect evidence of the presence or absence of in vivo activity of *Prevotella* and *Capnocytophaga* IgA1 proteases, by detection of antibodies in serum directed at a neoepitope exposed at the cleavage site of the Fab fragment of IgA1. On the basis of this investigation, the sera could be divided into two groups based on the previously indirectly demonstrated presence or absence of in vivo activity of *Prevotella* and *Capnocytophaga* IgA1 proteases (Table 2). A higher proportion of the sera with evidence of in vivo activity inhibited the *P. denticola* protease ($p = 2.2\%$, Fisher's exact test). No significant differences with regard to inhibition titer were observed (Wilcoxon's test). The sera represented different categories of periodontal disease as well as control subjects. No correlation to periodontal disease status was observed with regard to the presence or absence or titer of inhibitory activity towards any of the proteases (Table 2).

TABLE 2. Presence and titers of inhibition of *Prevotella* IgA1 proteases in human sera

Subject type	Source of IgA1 protease					
	P. buccae CCUG 93811	*P. oris* CCUG 15405	*P. buccalis* CCUG 15557	*P. denticola* CCUG 15558	*P. loescheii* CCUG 5914	*P. melaninogenica* ATCC 25845^T
All individuals ($n = 34$)	31/7 (5–19)[a]	34/22 (8–55)	3/6 (5–9)	4/6 (5–7)	33/8 (5–16)	9/5 (5–19)
Individuals with evidence of in vivo activities of IgA1 proteases[b]						
Present ($n = 14$)	13/7 (5–10)	14/22 (8–41)	1/5	4/6 (5–7)	14/7 (5–16)	6/5 (5–19)
Absent ($n = 20$)	18/6 (5–19)	20/22 (9–55)	2/8 (6–9)	0	19/9 (5–13)	3/5 (5–16)
Juvenile controls ($n = 5$)	5/8 (6–17)	5/20 (17–55)	1/5	1/7	5/10 (7–12)	1/9
Adult controls ($n = 7$)	7/7 (5–15)	7/30 (10–43)	1/6	1/5	7/8 (6–9)	3/5 (5–16)
Juveniles with periodontitis ($n = 7$)	7/6 (5–19)	7/25 (9–44)	0	0	7/10 (8–11)	0
Individuals with rapidly progressive periodontitis ($n = 8$)	6/7 (5–8)	8/19 (10–41)	0	2/6 (5–6)	8/8 (6–16)	3/5 (5)
Adults with periodontitis ($n = 7$)	6/6 (5–7)	7/20 (8–27)	1/7	0	6/7 (5–8)	1/12 (5–19)

[a] Number of sera that inhibited protease (as defined in Materials and Methods)/median titer of inhibition (range of titers). Titer is defined as the serum dilution giving rise to 50% inhibition of 1 U of IgA1 protease activity (see Materials and Methods).

[b] Previously demonstrated (11).

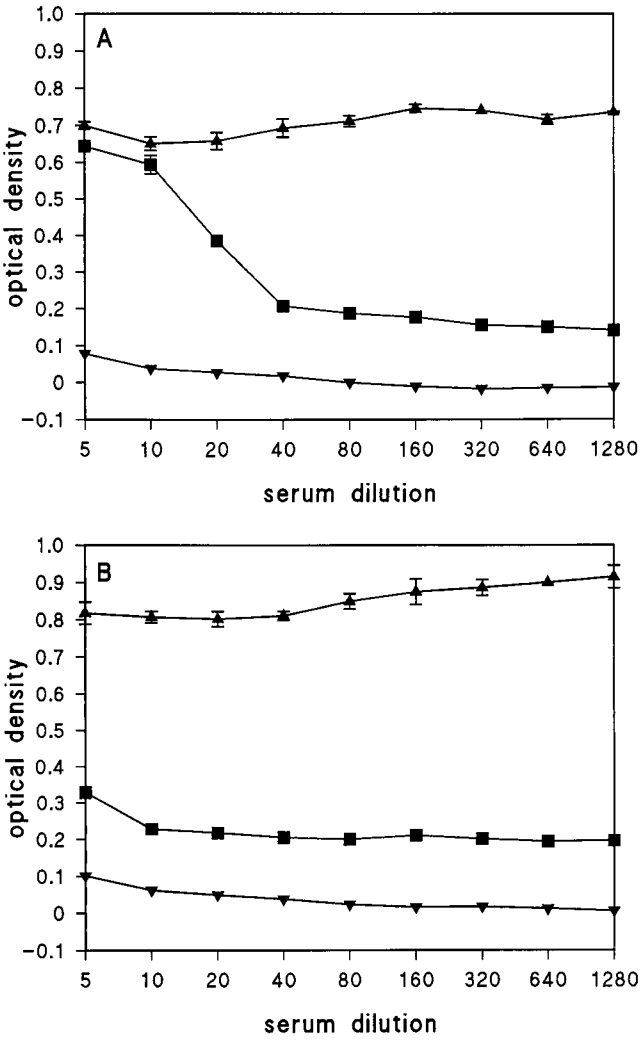

FIG. 2. Titration of sera for inhibitory activity against *P. melaninogenica* ATCC 25845^T IgA1 protease as described in Materials and Methods. ▲, mean (range of duplicates) ODs of the intact substrate control (titrated serum plus myeloma IgA1 Kah); ▼, background response (titrated serum plus myeloma IgA1 reaction with coating layer only); ■, mean (standard deviation of triplicates) ODs of the reaction mixture (titrated serum preincubated with 1 U of protease to which myeloma IgA1 Kah was subsequently added). (A) Serum with an inhibition titer of 16; (B) serum recorded to present no inhibition.

The relatively low titers of inhibition in almost all sera of *P. buccae*, *P. oris*, and *P. loescheii* proteases could suggest that serum components other than antibodies took part in the inhibition. This possibility was investigated further. Among the 12 sera showing the highest titers of inhibition of at least one of these three proteases, only 2 sera had a concomitantly high titer of inhibition of one of the other proteases. Eleven sera inhibited either *P. denticola*, *P. melaninogenica*, or *P. buccalis* IgA1 proteases, and only three sera concomitantly inhibited one more protease, while one serum inhibited all three proteases. Three sera from individuals with hypogammaglobulinemia (reduced levels of both IgG, IgA, and IgM) (Table 3) were tested for their ability to inhibit the *P. buccae*, *P. oris*, and *P. loescheii* proteases. One serum inhibited only the *P. oris* protease, while the two other sera inhibited all three proteases, with titers of the same magnitude as for sera with normal Ig levels (Table 3). Fractionation of one serum (serum 42) by size exclusion chromatography revealed that the inhibitory activity against *P. buccae*, *P. oris*, and *P. loescheii* was found in the Ig-containing fractions (Fig. 3). Inhibition of all three proteases was observed in the fractions containing the peak volume of IgA. In addition, inhibition of *P. oris* IgA1 protease was also recorded in some fractions containing both IgA and IgG. These data were supported by purifying the IgG fraction of serum 42 on a protein G column and subsequently recovering IgG antibodies against the *P. oris* enzyme only (Table 4). Purification of the IgG fraction from an additional four sera revealed inhibitory IgG antibodies against all three IgA1 proteases, the highest titer being always for the *P. oris* enzyme (Table 4). For both *P. buccae* and *P. oris*, the observed inhibition was of the same magnitude as for full sera.

TABLE 3. Inhibition titers in three hypogammaglobulinemic sera against selected *Prevotella* IgA1 proteases

Serum	Concn (mg/ml) in serum[a] of:			Titer of inhibition of IgA1 protease[b] from:		
	IgG	IgA	IgM	*P. buccae* CCUG 93811	*P. oris* CCUG 15405	*P. loescheii* CCUG 5914
0858	1.9	0.6	0.4	0	17	0
320613	1.3	0.2	0.2	6	28	6
750919	2.1	0	0	6	20	5

[a] Ranges of concentrations of IgG, IgA, and IgM in normal serum are 8 to 16, 1.4 to 4, and 0.5 to 2 mg/ml, respectively.

[b] Titer is defined as the serum dilution giving rise to 50% inhibition of 1 U of IgA1 protease activity (see Materials and Methods). Ranges of titers of inhibition of IgA1 proteases (medians) from *P. buccae* CCUG 93811, *P. oris* CCUG 15405, and *P. loescheii* CCUG 5914 in normal serum are 5 to 19 (7), 8 to 55 (22), and 5 to 16 (8), respectively. These data are from Table 2.

DISCUSSION

Low titers of inhibition of *Prevotella* IgA1 proteases in sera from both periodontally diseased and healthy individuals was observed in our study by using a recently published method for measurement of serum- and saliva-induced inhibition of IgA1 proteases (38). The titratable backgrounds of some sera with the coating layer (rabbit anti-mouse antibodies) were reported by Reinholdt to disappear upon replacement of the antibody with the same antibody in the form of $F(ab')_2$. Reinholdt explained this phenomenon by rheumatoid factor activity. Some of our sera produced titratable background responses even with the $F(ab')_2$ form of the coating antigen, but the reaction was minimized by using the FITC-conjugated $F(ab')_2$ form (Fig. 1). It remains an open question whether the FITC molecules [mean of 2.3 per $F(ab')_2$ molecule] masked a binding site for antibodies in serum or whether the effect was due to the increased hydrophobicity of $F(ab')_2$, which is the main chemical change in the molecule upon conjugation (16a). The amount of each serum available precluded testing of all *Prevotella* and *Capnocytophaga* IgA1 proteases, but instead, comparison between the presence or absence of serum-induced inhibition and previously demonstrated evidence of the in vivo activities of these same enzymes (11) was possible.

The antibody nature of the serum-induced inhibition of *Prevotella* IgA1 proteases was suggested by the different inhibitory patterns observed with the respective enzymes, which apart from a previously observed antigenic diversity, have identical properties. They all cleave the proline-serine bond between residues 223 and 224 in the IgA1 hinge region and belong to the same class of proteinases (9, 21, 34). A high titer of inhibition of one protease did not correlate with high titers of

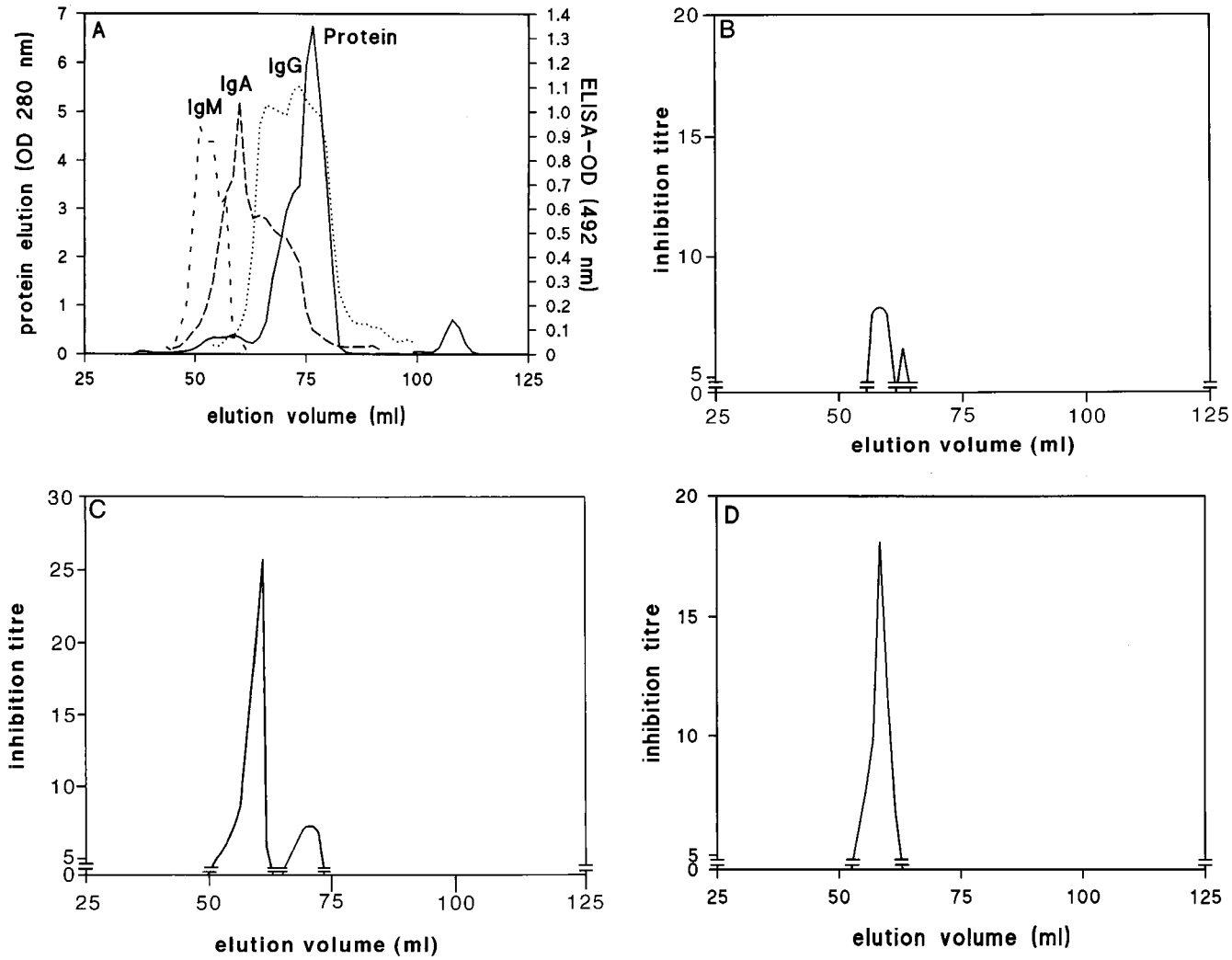

FIG. 3. Inhibition of *P. buccae*, *P. oris*, and *P. loescheii* IgA1 proteases by a normal human serum (serum 42) fractionated on a size exclusion column (Superose 6B). The protein profile is shown together with ELISA OD profiles of IgM, IgA, and IgG (A). Inhibition titers as defined in Materials and Methods are shown against the IgA1 proteases of *P. buccae* (B), *P. oris* (C), and *P. loescheii* (D).

inhibition of other proteases. Moreover, subjecting serum to size exclusion chromatography revealed that the inhibitory activity against the three IgA1 proteases that were inhibited by all tested sera coeluted with the Ig-containing fractions (Fig. 3). Furthermore, IgG isolated from five sera inhibited two of the three proteases to the same extent as was observed with full serum containing comparable amounts of IgG (Table 4).

The presence of a nonspecific inhibitor could not be ruled out in this study, as three hypogammaglobulinemic sera also inhibited the same three IgA1 proteases (Table 3). The inhibition may have been due to antibodies, but it is then puzzling that the reduced levels of IgA and IgG were not reflected in reduced titers of inhibition compared to those of normal sera. However, the Ig levels in these sera were only a 2- to 3-$\log_2$ dilution step away from normal serum levels, which therefore does not place these findings out of the range of normal inhibition titers. Neither α_2-macroglobulin nor α_1-proteinase inhibitor inhibits *Prevotella* IgA1 proteases (9, 34), and a potential unknown nonspecific inhibitory substance must coelute with the Ig-containing fractions to be in accordance with the results presented in Fig. 3.

TABLE 4. Titers of inhibition of selected *Prevotella* IgA proteases in sera and purified IgG fractions from five individuals

Individual	Sample type	Titer[b] of inhibition of IgA1 protease from:		
		P. buccae CCUG 93811	*P. oris* CCUG 15405	*P. loescheii* CCUG 5914
41	Serum	5	17	9
	IgG[a]	7	17	0
42	Serum	<5[c]	13	7
	IgG	<5	9	0
43	Serum	<5	18	10
	IgG	6	15	0
44	Serum	<5	27	16
	IgG	9	27	9
45	Serum	6	31	10
	IgG	12	35	<5

[a] IgG fractions were purified by passage on a protein G column and adjusted to the same IgG concentration as in serum.

[b] Titer is defined as the serum dilution giving rise to 50% inhibition of 1 U of IgA1 protease activity (see Materials and Methods).

[c] <5, inhibition was observed, but it was below the threshold defined by footnote *b*.

Our study demonstrated a low level of systemic inhibitory antibodies towards *Prevotella* IgA1 proteases (Table 3). The lack of inhibition of the *C. ochracea* CCUG 9716 protease and thus presumably IgA1 proteases from all members of this species (9, 13) was surprising, because antibodies reacting with cells of *Capnocytophaga* species may be found in periodontally diseased subjects (6, 43). However, little or no neutralizing antibody activity in serum against IgA1 proteases of other commensal organisms such as *Streptococcus oralis* and *Clostridium ramosum* has been reported for healthy individuals (14, 15, 25), whereas high titers of neutralizing antibodies in sera may develop if oral streptococci are involved in extraoral infections such as subacute endocarditis (39). The lack of inhibition or low titer of inhibition of *Prevotella* and *Capnocytophaga* IgA1 proteases may reflect a poor responsiveness of the immune system to these commensal bacteria unless they invade the tissues.

Sera inhibited *Prevotella* IgA1 proteases irrespective of evidence of in vivo activity. In vivo activity had previously been indirectly demonstrated by detecting in serum antibodies directed at a neoepitope exposed at the cleavage site of the Fab fragment of IgA1. As all these enzymes are of the same cleavage specificity, antibodies against this neoepitope may reflect in vivo activity of any or of a combination of the individual *Prevotella* and *Capnocytophaga* proteases. The presence of inhibitory antibodies despite the absence of evidence for in vivo activity may be explained by cross-reactive antibodies to other enzymes and by a longer persistence of enzyme-neutralizing antibodies than of anti-neoepitope antibodies in serum. The concomitant presence of inhibitory antibodies and evidence of in vivo activity conceivably means that IgA1 protease activity preceeded the development of inhibitory antibodies but may also mean that *Prevotella* or *Capnocytophaga* IgA1 proteases other than the ones for which we tested had been active at the time of sampling. Another explanation may be that the titer of inhibitory antibodies was not sufficient to effectively inhibit the proteases. If so, it might indicate that the IgA1 proteases of these bacteria may be active for a prolonged period of time.

The significance of *Prevotella* and *Capnocytophaga* IgA1 proteases in disease development has not been fully elucidated, but a clue to the understanding of the function of these enzymes may be that IgA1 protease-derived Fab fragments have been shown to retain full antigen-binding capacity (27, 28). Fab fragments of IgA1 antibodies binding to the bacterial surface may, therefore, block the access of intact antibody molecules of the same or other isotypes and of immunocompetent cells, thereby allowing the bacteria to avoid the effector functions mediated by antibodies of other Ig isotypes (21, 22). Since cleavage is not restricted to antibodies against the protease-secreting bacteria (1, 41), the IgA1 protease activity of, e.g., *Prevotella* and *Capnocytophaga* species may confer an ecological advantage to the subgingival microbial community harboring these bacterial species. Several lines of evidence suggest that IgA1 exerts its protective functions without the inflammatory side effects mediated by the other Ig isotypes (19, 23). The IgA1 proteases of *Prevotella* and *Capnocytophaga* species may therefore selectively disarm the nonphlogistic part of the human adaptive immune system operating in the periodontal area. This may conceivably augment inflammatory reactions and increase the risk of periodontal destruction.

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