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Comparative Analysis of Immunoglobulin A1 Protease Activity among Bacteria Representing Different Genera, Species, and Strains

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Immunoglobulin A1 (IgA1) proteases cleaving human IgA1 in the hinge region are produced constitutively by a number of pathogens, including *Haemophilus influenzae*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, and *Streptococcus pneumoniae*, as well as by some members of the resident oropharyngeal flora. Whereas IgA1 proteases have been shown to interfere with the functions of IgA antibodies in vitro, the exact role of these enzymes in the relationship of bacteria to a human host capable of responding with enzyme-neutralizing antibodies is not clear. Conceivably, the role of IgA1 proteases may depend on the quantity of IgA1 protease generated as well as on the balance between secreted and cell-associated forms of the enzyme. Therefore, we have compared levels of IgA1 protease activity in cultures of 38 bacterial strains representing different genera and species as well as strains of different pathogenic potential. Wide variation in activity generation rate was found overall and within some species. High activity was not an exclusive property of bacteria with documented pathogenicity. Almost all activity of *H. influenzae*, *N. meningitidis*, and *N. gonorrhoeae* strains was present in the supernatant. In contrast, large proportions of the activity in *Streptococcus*, *Prevotella*, and *Capnocytophaga* species was cell associated at early stationary phase, suggesting that the enzyme may play the role of a surface antigen. Partial release of cell-associated activity occurred during stationary phase. Within some taxa, the degree of activity variation correlated with degree of antigenic diversity of the enzyme as determined previously. This finding may indicate that the variation observed is of biological significance.

Bacterial immunoglobulin A1 (IgA1) proteases are endopeptidases capable of cleaving human IgA1, including the secretory form (S-IgA1), in the hinge region. Thereby the antibody molecules are left as intact Fab_{α} (monomeric) fragments devoid of the Fc_{α}, or $(Fc_{\alpha})_2 \cdot SC$, portion, which is particularly responsible for the protective properties of this immune factor (20). IgA1 protease is produced constitutively by a number of pathogens, including *Haemophilus influenzae*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, and *Streptococcus pneumoniae* and by several resident bacteria of the oral cavity and the upper airways. Among these are *Streptococcus mitis* biovar 1, *Streptococcus oralis*, and *Streptococcus sanguis*, which have attracted attention particularly because of their ability to initiate the formation of dental plaque (37). Moreover, *S. mitis* biovar 1 in the pharynx of infants may play a role in atopic sensitization (18). IgA1 protease-producing bacteria of the oral flora also include species of *Prevotella* and *Capnocytophaga*, which are of relevance to periodontal disease (17). Comprehensive lists of bacteria with IgA1 protease activity are provided by several reviews (21, 35, 38).

Three of the four different catalytic principles identified among proteolytic enzymes are represented among these proteases. The IgA1 proteases of *H. influenzae* and the pathogenic *Neisseria* species are of the serine type (2), those produced by streptococcal species are of the metallo type (10, 44, 55), and a cysteine type of protease is produced by *Prevotella* species (34). Thus, unrelated bacteria seem to have acquired this highly specific enzymatic activity by convergent evolution. This observation provides strong indirect evidence for the functional importance of these enzymes. IgA1 proteases of taxonomically different bacteria also differ with respect to the exact site of cleavage within the substrate. Individual enzymes cleave one of several prolyl-seryl or prolyl-threonyl bonds within the duplicated octapeptide, which constitutes the hinge region of human IgA1 and often next to a carbohydrate side chain (21). More than one type of enzyme has evolved within *H. influenzae* and the pathogenic neisseriae. For these species, cleavage between proline and serine is designated type 1 activity, whereas cleavage between proline and threonine is designated type 2 activity. In addition to cleaving IgA1 of humans, the serine-type IgA1 proteases are involved in autoprocessing during their secretion through the gram-negative outer membrane (41, 43). Cleavage of a few other proteins of bacterial and human origin has been observed under experimental conditions (14, 32, 52).

Analyses of IgA1 protease-encoding genes (*iga*) and their translation products have revealed homology within the group of serine-type proteases and within the group of metallo-type proteases produced by streptococcal species (26, 42), thus confirming the distinct evolutionary lineages of these enzymes. However, cross-inhibition studies with specific antisera have shown considerable antigenic diversity of the enzymes not only across but also within some species boundaries, possibly reflecting immunological selection pressure (26, 27).

In vitro experiments have indicated that cleavage of S-IgA1 by IgA1 proteases interferes with most of the protective functions mediated by antibodies of this isotype (20). Still, the exact biological significance of IgA1 proteases remains enigmatic (22). Cleavage of IgA1 of human secretions in vivo has been demonstrated (1, 53) but may eventually require substantial amounts of IgA1 protease to overcome the effects of induced protease-neutralizing antibodies. Antibodies to IgA1 proteases have been detected in serum as well as in secretions (6, 9, 48, 54) and may be induced not only during invasive infection but also during a healthy state of carriage, as demonstrated in the

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case of *N. meningitidis* (4). However, shifts in the flora toward significantly higher numbers of IgA1 protease-producing bacteria or bacteria producing an antigenic type of protease not previously encountered by the host (30) may conceivably result in local impairment of the immune barrier to microbial and other antigens, including potential allergens (18). Isolated observations of substantial differences in the amount (50) and immunogenicity (9, 47) of IgA1 proteases produced by certain pathogens and commensals suggest that this possibility may not apply equally to all IgA1 protease-producing bacteria.

A study by Plaut and coworkers (40) added further complexity to this picture. They found that human milk antibodies neutralizing serine-type IgA1 proteases interfered with the autoproteolytic mechanism of release of these enzymes and that IgA1 protease molecules consequently persisting on the cell surface provided for antibody-mediated agglutination of the bacteria. Thus, cell-bound and secreted IgA1 protease may have distinct roles in vivo.

Assuming that the biological role of individual IgA1 proteases may be related to the levels of total and cell-bound protease generated, we have compared IgA1 protease activities in cultures of 38 bacterial strains representing different genera and species as well as strains of different pathogenic potential. This was accomplished by using a protocol for determination of IgA1 protease activity generation rate involving repetitive measurement of secreted and cell-associated activity during culture.

MATERIALS AND METHODS

Bacteria and media. The 38 bacterial strains analyzed are listed according to taxonomic status in Table 1, which also includes information on the original isolation of the strains. The production of IgA1 protease by each strain was documented in previous experiments using a highly sensitive assay. Briefly, one loopful of bacteria (approximately 10^9 organisms) from an appropriate solid medium was incubated with 40 μl of myeloma IgA1 at 1.5 mg ml^{-1} overnight at 37°C. Protease activity was subsequently revealed by the generation of two distinct precipitation lines corresponding to Fab_α and Fc_α upon immunoelectrophoresis against rabbit antibodies to intact human IgA (49). Also, cleavage types of the proteases from *H. influenzae*, *N. meningitidis*, and *N. gonorrhoeae* had been previously determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1) of cleaved myeloma IgA1 (Table 1).

For quantitative analysis, the bacteria were grown in liquid medium, allowing for numerical characterization of the bacteria responsible for measured quantities of protease activity. Strains of *H. influenzae*, *N. meningitidis*, *N. gonorrhoeae*, or *S. pneumoniae* were grown in Levinthal broth at 37°C in an atmosphere of air plus 5% CO_2, using a CO_2 incubator. Strains of *S. mitis*, *S. oralis*, and *S. sanguis* were grown in Todd-Hewitt broth (Difco, Detroit, Mich.) under the same conditions. *Prevotella* and *Capnocytophaga* strains were grown in plaque medium (15) in an atmosphere of 80% N_2, 10% H_2, and 10% CO_2, using an incubator within an anaerobic glove box.

Quantitation of IgA1 protease activity. IgA1 protease quantitation experiments were done according to the following protocol, a maximum of four strains being analyzed in parallel. Fifteen milliliters of medium in 20- by 150-mm loosely capped Duran glass test tubes (Schott, Mainz, Germany) was inoculated with 0.3 ml of a 12-h preculture of the test strain. The medium had been equilibrated by overnight incubation in the prospective culture atmosphere at 37°C. During the following 7 to 9 h, the growing bacteria were kept in suspension by frequent manual agitation of the tube within the incubator. At appropriate intervals, the density of the culture was measured in terms of optical density (OD) at 490 nm, using a Spectronic 20 spectrophotometer (Bausch & Lomb, Rochester, N.Y.) equipped with a light cell fitting the culture tube. At each of these occasions, a 1-ml sample was withdrawn for determination of IgA1 protease activity and, in some cases, counting of cells by microscopy (see below). Sampling of *Prevotella* and *Capnocytophaga* cultures was done within the anaerobic glove box. Generally, OD measurement and sampling were completed within 2 min, after which the culture was replaced in the incubator. A final OD measurement and sampling was done after incubation overnight (24-h sample). At this point, a small volume of the remaining culture volume was inoculated on homologous solid medium to check for possible contamination. Immediately after isolation, samples received 30 μg of chloramphenicol (12 μl of a 0.25% solution in water) to arrest production of bacterial proteins, including IgA1 protease. This amount corresponds to at least 20 times the MIC for the bacteria studied (8) but did not affect the activity of IgA1 proteases, as demonstrated by comparative analysis of culture supernatants with and without chloramphenicol (results not shown). The sample supernatant, isolated after centrifugation at 2,000 × *g* for 3 min, was transferred to a separate tube. The bacteria in the pellet were then washed twice, each time by brief vortexing in 1.5 ml of cold, chloramphenicol-containing 0.05 M phosphate-buffered saline (PBS) at pH 7.4 or 5.5 (see below). The last volume of washing buffer from samples collected at early stationary phase was saved for control of washing efficiency. The bacteria were resuspended in 1 ml of PBS and kept at 4°C until analyzed the following day.

In the cases of six strains representing individual species (Table 1), the convenient recordings of culture density by OD were supplemented by corresponding direct cell counts as obtained by phase-contrast microscopy at a magnification of ×630, using a Helber counting chamber (Scherf Präzision, Ostheim-Urspringen, Germany). Total counts for individual samples were based on enumeration of approximately 200 cells, high-density samples being counted at an adequate degree of dilution. Bacteria in high-density samples of the *Capnocytophaga sputigena* culture were disaggregated prior to dilution and counting by sonication for 5 s at 25 W in a B-12 sonifier (Branson, Danbury, Conn.). All samples from the six strains were counted by one and the same reader. ODs recorded for the other strains were subsequently transformed into the form of cell number per milliliter by reference to a regression curve fitted to a plot of counts against OD for the reference strain of the relevant species. Strains belonging to either of the three closely related species *S. mitis*, *S. oralis*, and *S. sanguis* displayed indistinguishable morphological characteristics, including chain length. Hence, a strain of *S. sanguis* (SK1) served as the common counting reference for these species. Likewise, *N. meningitidis* HF13 served as the common reference for *N. meningitidis* and *N. gonorrhoeae.*

IgA1 protease activity in culture supernatants, cell suspensions, and washing buffer controls was measured by titration using a previously described assay (45). Briefly, serial twofold dilutions (50 μl) of samples were incubated with 50 μl of a solution of purified myeloma IgA1 substrate (20 μg ml^{-1}) in sealed microtiter plates, the absorptive capacity of which had been blocked by prior incubation with a 0.15% solution of Tween 20. The diluent of samples as well as of substrate IgA1 was PBS of a pH optimal for the IgA1 protease activity of the species (pH 5.5 for *Prevotella melaninogenica* and *C. sputigena*; pH 7.4 for all others [21]). The plates were incubated at 35°C for 6 h while attached to a rotating table operated at 200 rpm. Protease-induced cleavage of IgA1 in each well was subsequently measured by enzyme-linked immunosorbent assay as described previously (45), providing for calculation of IgA1 protease activity in undiluted samples in terms of C_{50} units per milliliter, C_{50} referring to 50% cleavage of substrate (45). Occasionally, bacterial suspensions and supernatants isolated during early growth phases were unable to cleave 50% of the substrate. The activity of such samples was quantitated by reference to the enzyme-linked immunosorbent assay titration curve of a homologous sample of adequate potency analyzed on the same plate.

The IgA1 protease activity of each strain was expressed as the rate of activity generation during growth calculated on a per-cell basis. How this quantity, designated A_{gr} (for activity generation rate), was derived from the data of the sequentially collected samples is illustrated in the case of *S. mitis* SK677 (Fig. 1). The growth curve of the strain was constructed by fitting an asymmetrical sigmoid model (Fig. P software package; Biosoft, Cambridge, England) to a plot of total cell counts per milliliter (N) against culture time (t). Two samples, corresponding to early exponential (sample 1) and early stationary (sample 2) phases, were identified by reference to the growth curve (Fig. 1). Let $N(t)$ be the function of the growth curve, let $A1_{sup}$ and $A1_{cel}$ be the measured activities in supernatant and cells of sample 1, respectively, and let $A2_{sup}$ and $A2_{cel}$ be the corresponding activities of sample 2. Provided that the amount of protease released from the bacteria during washing was negligible and assuming that A_{gr} is constant within the observation period t_1 to t_2, we can now write $A2_{sup} + A2_{cel} = A1_{sup} + A1_{cel} + \int_{t_1}^{t_2} A_{gr} \times N(t)dt$ or $A_{gr} = (A2_{sup} + A2_{cel} - A1_{sup} - A1_{cel}) \times \int_{t_1}^{t_2} N(t)dt^{-1}$. The integral $\int_{t_1}^{t_2} N(t)dt$ was calculated by application of the area-under-the-curve function of the Fig. P software package to the relevant segment of the growth curve (Fig. 1).

The IgA1 protease activity in cultures of individual strains was further characterized by recording the total activity accumulated until early stationary phase ($A2_{sup} + A2_{cel}$) and the proportion of the accumulated activity that was cell associated ($A2_{cel} \times [A2_{sup} + A2_{cel}]^{-1}$). Different bacteria were compared with respect to cell-associated IgA1 protease activity on the basis of activity associated with a standard number of 10^8 cells at early stationary phase ($A2_{cel} \times N[t_2]^{-1} \times 10^8$). Additional calculation of the activity associated with 10^8 cells at late stationary phase (24 h) provided for determination of the change, if any, in cell-associated activity during stationary phase.

RESULTS

Methodological observations. The bacteria grew well in their respective media, producing sigmoid growth curves as illustrated for three strains of *S. mitis* (Fig. 1).

IgA1 protease activity was characterized by separate quantitation of secreted and cell-associated activity in culture samples. To quantitate the cell-associated fraction, the isolated cells were washed prior to being analyzed. The protease activ-

TABLE 1. IgA1 protease activities in cultures of 38 bacterial strains characterized with respect to taxonomic affiliation, IgA1 protease cleavage type, and history of pathogenicity[a]

Species and strain	Serogroup, type, biogroup, or biovar	IgA1 protease cleavage type[b]	Source isolated or obtained from	A_{gr} (U cells^{-1} min^{-1}) (10^{11})	Activity at early stationary phase			Decline in cell-associated activity during stationary phase (%)
					Accumulated in supernatant + cells (U ml^{-1})	Cell associated (%)	Calculated/10^8 cells (U)	
H. influenzae								
HK 368	b	1	Meningitis	18	52	7.6	0.3	>90
HK 393[c]	b	1	Meningitis (=NCTC 8467)	34	329	1.4	0.1	>90
HK 635	c	2	Bronchitis	39	389	6.2	0.5	>90
HK 224	NC	2	Otitis media	46	99	5.1	0.2	>90
HK 284	NC	2	Conjunctivitis	1,332	12,830	2.3	1.1	>90
HK 869	Aegyptius	2	Brazilian purpuric fever	187	1,267	1.3	0.8	>90
N. meningitidis								
NK 183	A	1	Meningitis	502	5,910	1.0	0.4	>90
NGC 80	B	1	Healthy carrier	806	16,560	0.5	0.5	>90
NG 117	B	2	Meningitis	1,836	41,080	0.3	0.7	>90
HF 13[c]	Y	2	Meningitis	2,054[d] (2,290, 1,818)	70,855 (72,260, 69,450)	0.1 (0.1, 0.1)	0.3 (0.3, 0.3)	>90 (>90, >90)
N. gonorrhoeae								
BK 41		1	Gonorrhoea	251	712	0.0	0.0	ND[e]
NG 74		1	Gonorrhoea	39	188	0.0	0.0	ND[e]
BK 48		2	Gonorrhoea	429	4,100	1.2	0.8	ND[e]
BK 42		2	Gonorrhoea	431	7,950	0.9	0.9	ND[e]
S. pneumoniae								
SK 676[c]	7F		Sepsis, adult	149	983	79	34	17
SK 674	NC		Pharynx, healthy adult	1,009 (1,128, 889)	5,066 (5,960, 4,172)	45 (50, 40)	112 (128, 96)	86 (89, 83)
SK 675	NC		Pharynx, healthy adult	105	670	52	18.5	81
NCTC 7465	1		NCTC	314	817	62	16	75
PK 81	14		Clinical isolate	206	790	69	14	ND[e]
PK 82	14		Clinical isolate	79	292	57	7.4	ND[e]
PK 84	18C		Clinical isolate	62	275	55	0.2	ND[e]
S. mitis								
SK 605	1		Pharynx, atopic child	5.3	37	16	0.2	5
SK 607	1		Pharynx, atopic child	317	2,093	35	41	21
SK 569	1		Blood, neutropenia (Francioli)	168	1,022	32	16	48
SK 597	1		Urethra	291	1,103	36	33	50
SK 286	1		CCUG 25812	1.2	105	100	0.3	10
SK 656	1		Oral mucosa, healthy infant	0.5	5.2	100	0.2	ND
SK 644	1		Oral mucosa, healthy infant	0.9	6.1	100	0.2	ND
SK 677	1		Oral mucosa, healthy infant	69	873	11	3.4	76
S. oralis								
SK 10			SBE	3.4 (2.4, 4.3)	13 (17, 10)	100 (100, 100)	0.4 (0.4, 0.3)	6 (12, 0)
SK 562			SBE	12	65	69	1.3	73
SK 23			Oral cavity (=NCTC 11427)	6.4 (9.2, 3.6)	67 (82, 51)	64 (70, 59)	1.3 (1.6, 0.9)	7 (14, 0)
S. sanguis								
SK 1[c]	1		SBE (=ATCC 10556)	12 (15, 9.8)	70 (102, 37)	57 (48, 66)	1.1 (1.4, 0.8)	56 (50, 62)
SK 4	2		Dental plaque, Carlsson, 804	260	2,097	17	10	74
SK 160	3		Initial dental plaque	68	388	21	2.4	88
SK 678	ND		SBE	166	958	16	4.5	92
P. melaninogenica ATCC 28845[c]			ATCC	9.6	65	62	2.3	ND
C. sputigena ATCC 9714[c]			ATCC	3.6	14	55	0.4	ND

[a] ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures; CCUG, Culture Collection at the University of Gothenburg, Gothenburg, Sweden; NC, noncapsulated; SBE, subacute bacterial endocarditis; ND, not determined.
[b] Relevant for *H. influenzae*, *N. meningitidis*, and *N. gonorrhoeae* only.
[c] Strain used as counting reference.
[d] Mean of two determinations (first, second).
[e] Irrelevant due to bacteriolysis at 24 h.

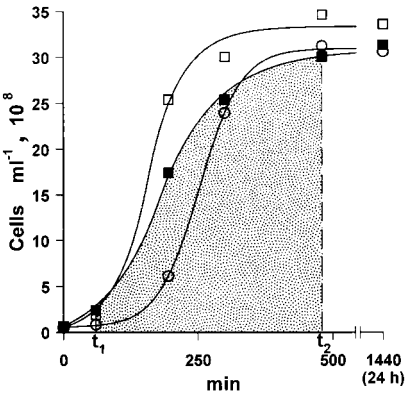

FIG. 1. Growth curves for *S. mitis* SK656 (□), SK677 (■), and SK644 (○). In the case of strain SK677 are indicated the sampling times for the early-exponential-phase (t_1) and the early-stationary-phase (t_2) samples involved in the calculation of A_{gr} and the area (shaded) corresponding to the integral $\int_{t_1}^{t_2} N(t)dt$.

ity measured in the last volume of washing buffer was negligible except for a few *H. influenzae* and *Neisseria* strains, where it was approximately 10% of the very limited activity measured for the cells of these bacteria (Table 1). Thus, contaminating supernatant introduced no significant bias in the quantitation of cell-associated activity.

To examine the reproducibility of results, five strains were analyzed twice. The five strains were selected upon the first analysis to represent the extreme variation observed with respect to total activity and its distribution between supernatant and cells (Table 1). The two analyses were independent except for the use of a common reference curve for the transformation of OD readings to cell numbers. The duplicated experiments indicated high reproducibility for strains of high activity irrespective of a large (SK674) or small (HF13) proportion of cell-associated activity (Table 1). Quantitation of the low and often largely cell-associated activity of certain streptococci (SK10, SK23, and SK1) was less reproducible, as suggested by a ratio of up to 2.6 for the largest relative to the smallest of two determinations of A_{gr} (Table 1). In view of these observations, differences in A_{gr} corresponding to less than a factor of 2 should be interpreted as not significant when the activities of strains are compared. Presumably, the limited reliability of the assay in measuring very small quantities of IgA1 protease was responsible for the exclusive detection of cell-associated activity in cultures of four strains of commensal streptococci (SK286, SK656, SK644, and SK10). IgA1 protease preparations from these strains have been previously prepared by concentration of culture supernatants (46).

Taxon- and strain-related variations in A_{gr}. The results for all bacteria are compiled in Table 1. The highest levels of activity were recorded for two strains of *N. meningitidis* (HF13 and NG117). These strains generated per minute roughly 4,000 times the activity generated by the least active strain (*S. mitis* SK656). Concerning the pathogenic species, most strains of *N. meningitidis* (including an isolate from a healthy individual) and *N. gonorrhoeae* were of relatively high activity, whereas activities of strains of *H. influenzae* and *S. pneumoniae* varied widely. Notably, two pathogenic *H. influenzae* serotype b strains isolated from cases of meningitis were of lower activity than two noncapsulated strains. Also, within *S. pneumoniae*, high activity was not a preferential property of capsulated clinical isolates. Among the commensal streptococci, large intraspecies variation was observed in *S. mitis* and *S. sanguis*, the most active strains exceeding the activity level of several *S. pneumoniae* strains. Three strains representing *S. oralis* were of low activity, as were the single strains of *P. melaninogenica* and *C. sputigena*. Among four strains of commensal streptococci having caused subacute bacterial endocarditis, three displayed low A_{gr} whereas one was of intermediate activity.

Among strains of *H. influenzae*, *N. meningitidis*, and *N. gonorrhoeae*, those producing IgA1 protease of cleavage type 2 displayed higher A_{gr} values than those producing type 1 protease. Yet, among four *H. influenzae* strains producing type 2 protease the activity varied considerably, two being at the level of the type 1 strains. A comparable situation was observed in *N. gonorrhoeae.*

Among four strains of *N. gonorrhoeae*, two producing type 2 IgA1 protease grew faster and reached higher (threefold) densities than two producing type 1 IgA1 protease, corroborating previous observations (50). No correlation of growth characteristics to type of IgA1 protease produced was observed among strains of *N. meningitidis* or *H. influenzae*. The pattern observed for *N. gonorrhoeae* that the strains with the highest growth rates (in that case the type 2 IgA protease-producing strains) also displayed the highest A_{gr} values did not apply to any other species (data on growth rates not shown).

The variation in A_{gr} was reflected in the accumulated activity (Table 1). This quantity is presented because it is of relevance in the production of IgA1 protease preparations for experimental purposes.

Cell-associated IgA1 protease activity. Cell-associated activity was detected in the early-stationary-phase cultures of almost all bacteria. In strains of *H. influenzae*, *N. meningitidis*, and *N. gonorrhoeae*, the activity of the cells constituted but a small fraction of the total accumulated activity. In contrast, the cells accounted for a significant fraction, and occasionally all, of the activity produced by strains of the four streptococcal species, *P. melaninogenica*, and *C. sputigena*. Significant species- and strain-related differences were evident also when cell-associated activity was calculated in units for a standard number of 10^8 cells. Expressed in this way, the cell-associated activity of streptococci (all four species included) at early stationary phase correlated positively with total activity in terms of A_{gr} (Spearman's $r = 0.94$, $P < 0.001$). Similar correlation was not observed within the group of *H. influenzae*, *N. meningitidis*, and *N. gonorrhoeae.*

Comparison of results for samples collected at early stationary phase and after 24 h revealed almost complete loss of cell-associated activity in cultures of *H. influenzae* and *N. meningitidis*. Bacteriolysis in 24-h-old cultures made this analysis irrelevant for strains of *N. gonorrhoeae* and three strains of *S. pneumoniae*. Reduction in cell-associated activity during stationary phase, though less pronounced, was observed also for strains of the four streptococcal species. The reduction was generally accompanied by an increase in the IgA1 protease activity of the supernatant, suggesting that the phenomenon was due to release of protease from the cells rather than inactivation of enzyme.

DISCUSSION

In this study, IgA1 protease activity of individual bacteria was quantitated on a per-cell basis in terms of A_{gr}. In deriving the mathematical expression of A_{gr}, it was assumed that the bacteria produced IgA1 protease at a constant rate from early exponential to early stationary phase, i.e., corresponding to the interval from t_1 to t_2 (Fig. 1). Whether this assumption was justified might have been examined by using a modification of the protocol employed, but this issue was beyond the scope of the study. Should bacteria be identified for which this assump-

tion is unjustified, A_{gr}, calculated as described in Materials and Methods, can still be regarded as an adequate measure of the IgA1 protease activities of such bacteria.

The activities measured in sequential culture samples (not shown) indicated that in almost all cultures, the accumulation of activity ceased once transition into stationary phase was completed. Hence, A_{gr} values are likely to be biased if calculated on the basis of a sampling period reaching into stationary phase.

The initial treatment of samples with chloramphenicol immediately followed by separation of supernatant and cells provided for selective quantitation of secreted and cell-associated activity at the time of sampling. Because the specific activities of cell-associated and free IgA1 protease molecules have not been compared, however, the values for these activities may not exactly have reflected the distribution of IgA1 protease molecules between the two compartments.

Comparative data on IgA1 protease activity in a few strains of *N. gonorrhoeae* have been previously reported by Simpson et al. (50). They found that the activity accumulated in an early-stationary-phase culture of a gonococcal strain producing type 2 protease was roughly 10 times the activity accumulated in cultures of various type 1-producing strains which, however, had grown to lower densities. Our analyses of gonococci corroborate these results and indicate, by the discrepant A_{gr} values, that the distinct levels of activity cannot be ascribed solely to the larger number of type 2-producing cells in the culture. In addition, our results indicate that a similar hierarchy concerning activity of type 2 and type 1 protease-producing strains applies not only to *N. meningitidis* but also to *H. influenzae*, irrespective of the fact that *H. influenzae* type 1 and neisserial type 1 enzymes cleave distinct prolyl-seryl peptide bonds (21).

Enzymological aspects of the activity variation. At present, we can only speculate on explanations to the different levels of IgA1 protease activity observed. Superior activity of some bacteria over others could be expected if they produced the protease at a higher rate or if their protease molecules were more efficient in cleaving the IgA1 substrate. The first possibility is purely theoretical, as no studies have quantitated IgA1 proteases except by activity, and aspects of relevance to the expression of *iga* genes have not been addressed in any detail. The second possibility is of interest in relation to the difference in activity of type 2 and type 1 protease-producing bacteria. In addition to involving distinct pairs of amino acids, the target peptide bonds of type 2 and type 1 proteases differ in proximity to glycans attached to the hinge region of the α1 chain (21). Previous studies, though with streptococcal IgA1 proteases, have indicated that these glycans influence the susceptibility of the protein to cleavage (49). However, the large variation in A_{gr} observed among type 2-producing *H. influenzae* and among type 1-producing *N. gonorrhoeae* (Table 1) indicates that cleavage specificity per se is at most one of several determinants of the level of IgA1 protease activity displayed by strains of *H. influenzae* and the two *Neisseria* species.

Data on the structure of IgA1 proteases from the three latter species are of interest in that context. These proteases share a common multidomain structure, a leader sequence and domains in the C-terminal half being involved in the particular secretion mechanism of the enzymes as originally demonstrated for *N. gonorrhoeae* (41). In the N-terminal half, sequence homologies are unevenly distributed, some regions being conserved and others showing a mosaic-like pattern of homologies suggesting horizontal genetic exchange (13, 29, 43). These sequence variations are reflected not only in the antigenic properties but also in the cleavage specificities of the enzymes (27, 29). The length and sequence of a particular region called the cleavage specificity determinant, which is distinct from the active site, seems to determine which of the two potential target bonds in the α1 chain will be attacked (12, 29). However, variations in the cleavage specificity determinant and neighboring regions of the molecule occur also among clones with identical cleavage specificity (29). It is tempting to speculate that such variation may affect the local steric relationship of the active site to the actual target bond and, hence, the efficiency of the enzyme. In fact, previous studies (28) showed sequence variations in these regions of the enzyme between the three type 2 protease-producing strains of *H. influenzae* (HK284, HK635, and HK869), which in the present study showed contrasting levels of activity (Table 1). Conversely, two type 2 protease-producing strains of *N. meningitidis* (HF13 and NG117), the enzymes of which were 96% identical in the same regions (29), were of closely similar activity (Table 1).

The strains of *H. influenzae* and the two *Neisseriae* species invariably displayed very little cell-associated IgA1 protease activity (calculated for a standard of 10^8 cells), all of which was eliminated during stationary phase. Thus, differences, if any, in the IgA1-cleaving activities of the proteases from these strains are not reflected in the efficiency of the autoproteolysis of the (nonglycosylated) preprotease molecule responsible for the extracellular release of the mature enzyme.

Marked variation in activity was observed also for strains of the four streptococcal species, the IgA1 proteases of which share a common ancestor and a cleavage specificity different from those of the type 1 and type 2 proteases of *H. influenzae* and the *Neisseria* (21). Three of these species, *S. pneumoniae*, *S. oralis*, and *S. mitis* (some strains), produce glycosidases, including neuraminidase, which are able to degrade the carbohydrate moiety of IgA1 molecules and thereby alter their susceptibility to cleavage by homologous IgA1 proteases (49). Species- and strain-related differences in glycosidase activity (3, 5, 19, 49) may have contributed to the variation in IgA1 protease activity observed, but other, unknown factors are likely to be responsible as well. The limited shifts in the susceptibility of IgA1 to cleavage after extensive degradation of its glycans (49) contrast with the extreme variation in A_{gr} observed, particularly among *S. mitis* strains (Table 1). Moreover, marked variation in A_{gr} was observed also among strains of the closely related species *S. sanguis*, which do not attack the glycans of IgA1 (49).

Hybridization analysis and sequencing of *iga* genes in *S. pneumoniae* (44, 55) supplemented with serological comparison of secreted proteins (25) have revealed a remarkable heterogeneity of pneumococcal IgA1 proteases, probably reflecting frequent genetic exchange between strains. Genetic and serological analysis of IgA1 proteases of commensal streptococci have revealed a similar extensive variation of the enzyme in *S. mitis* (42, 46), contrasting with very limited variation in *S. oralis* and *S. sanguis* (42, 49). Some of the streptococcal strains involved in these analyses were included in the present study (PK81, PK82, PK84, SK10, SK23, SK1, and SK4). However, no structural element of the enzyme covarying with A_{gr} could be identified.

Cell-associated activity of streptococci. The bacterial cells accounted for a significant fraction of the IgA1 protease activity in cultures of all streptococci (Table 1). The 57% cell-associated activity observed for *S. sanguis* ATCC 10556 contrasts with the level of 5% previously reported for cells of this strain (39). Conversely, the 55 to 78% cell-associated activity in cultures of pneumococci is below the level reported for one pneumococcal strain by Wani et al. (55). The latter discrepancy may reflect differences in the methods used, since Wani et al.

quantitated IgA1 protease not by activity but by its reaction in Western blots with a polyclonal rabbit antiserum raised by immunization with whole pneumococci (55). This antiserum reacted with the high-M_r form of the protease but apparently not with the lower-M_r forms which have been shown to occur in pneumococcal culture supernatants (44). Apart from being of immunological interest, the restricted reactivity of the antiserum used by these authors may have resulted in underassessment of the secreted form of the protease.

Streptococcal IgA1 proteases contain at the N terminus a sequence matching the C-terminal cell wall anchor common to many gram-positive bacterial surface proteins (42, 44, 55). Because of the atypical location of the anchor motif, previous observations concerning the function of the motif in anchoring proteins to the bacterial surface (36) may not apply in detail to streptococcal IgA1 proteases. The posttranslational (including postsecretional) cleavage of high-M_r streptococcal IgA1 protease into several smaller, still active molecular species which was originally detected in *S. pneumoniae* (44) has been observed also among the commensal streptococci (42). Whether such cleavage contributes to the release of IgA1 protease from the cells, and whether it reflects autoproteolysis as in the case of the serine type IgA1 proteases, is not clear (44). Anyhow, the notable activity of cells during growth suggest that streptococcal IgA1 proteases may play a role as surface antigens similar to that observed for the serine type of the enzymes in the presence of neutralizing antibodies (40). The observation that cell-associated IgA1 protease activity on a per-cell basis at early stationary phase correlates positively with A_{gr} and is released gradually during stationary phase suggests that one or more rate-limiting steps occur in the secretion process.

***Prevotella* and *Capnocytophaga* species.** The low and largely cell-associated IgA1 protease activities of two strains of *P. melaninogenica* and *C. sputigena*, respectively, are in accordance with previous observations of low levels of these proteases in culture supernatants (21, 34). The activities measured may have been influenced by concomitant glycosidase activity degrading IgA1 glycans (7). The structures of IgA1 proteases from *Prevotella* and *Capnocytophaga* species have not been examined, except that serological studies have revealed species-related variations in the antigenic properties of the enzymes (7).

The constitutive nature of IgA1 proteases. A pertinent question is whether the IgA1 protease activities measured in this study can be expected to reflect the activity that the same bacteria would generate during growth on mucosal surfaces or in tissues of the human host. IgA1 proteases have invariably been found to be constitutively produced in the sense that the activity is expressed during culture in a variety of media with or without content of human IgA1 (24). Interestingly, Shoberg and Mulks (51) reported that a clone of *N. gonorrhoeae* increased its production of protease during growth at iron-restricted conditions. The relevance of this observation to the in vivo situation may be questioned because gonococci (and several other bacteria with or without IgA1 protease activity) have developed means of acquiring iron in the iron-restricted environment of the human host (11, 31). However, this result and the conflicting data concerning potential IgA1 protease activity among members of the enteric flora (21, 33) call for reevaluation of the constitutive nature of IgA1 proteases.

Potential biological significance of variation in A_{gr}. Provided that the A_{gr} values measured reflect IgA1 protease activities as generated in vivo, it is interesting that all of the bacteria, irrespective of level of activity, had been able to colonize mucosal surfaces protected largely by IgA1 (16) (Table 1). The low A_{gr} values of two *H. influenzae* strains from meningitis patients indicate that invasive potential also is not restricted to bacteria with elevated activity. Our previous observation that *S. sanguis* SK1 cells become coated with Fab_α fragments during incubation in human saliva (1) indicates that a low IgA1 protease generation rate (A_{gr} for SK1 = 12) need not prevent bacteria from taking advantage of this enzymatic activity. Yet, the additional activity of certain bacteria, predominantly strains of pathogenic species, might be of biological significance. IgA1 protease-producing pathogens induce considerable levels of protease-neutralizing antibodies during colonization of mucosal membranes (4, 10, 48, 54). Because sharing of epitopes for neutralizing antibodies occurs among IgA1 proteases of related bacteria (26), such antibodies may interfere with the protease activity of subsequently acquired clonal variants. A new clone may colonize successfully if it possesses an antigenic type of protease distinct from those of previously colonizing clones (30). Conceivably, a clone producing an antigenic type sharing epitopes with proteases of previous clones might also succeed if it produces sufficient activity to overcome the effect of preexisting antibodies.

Some of the present results corroborate this hypothesis. Because antigenic variation among neisserial IgA1 proteases is very limited (26), a meningococcal or gonococcal clone encountering a new host is likely to have its protease activity reduced by antibodies raised by preceding clones of either of these species. The high and relatively uniform level of activity observed for the neisseria, except one gonococcal strain (Table 1), may indicate that the populations of these human pathogens have adapted to this situation. Conversely, the variable level of activity in *H. influenzae* and *S. pneumoniae* might reflect the extreme antigenic polymorphism of IgA1 proteases in each of these species.

Concerning the commensal streptococci, the wide range of activities within *S. mitis* makes sense in the view of the recently detected polymorphism of IgA1 protease in that species (46). Besides, the high activities of some *S. mitis* strains, including an isolate from the pharynx of an atopic child (SK607), corroborate the hypothesis that increased colonization with IgA1 protease-producing *S. mitis* in infants may compromise S-IgA-mediated protection and lead to atopic sensitization (18, 53). However, the low activity in *S. oralis* and the variable activity in *S. sanguis* are remarkable considering that the distinct IgA1 proteases of the two species are both antigenically homogeneous (49). Although *S. oralis*, *S. sanguis*, and *S. mitis* are permanent members of the oropharyngeal flora, neutralizing antibodies to their IgA1 proteases are not constantly present in all individuals (9, 46, 47). We suspect that elevated titers of antibodies regulating the IgA1 protease activity and the colonization of these streptococci may be induced by cross-reacting IgA1 proteases produced by certain clones of *S. pneumoniae* (27, 46).

The hypothesis that colonization by IgA1 protease-producing bacteria is influenced by the balance between preexisting, protease-neutralizing antibodies and the activity level of potentially colonizing clones may be examined by monitoring these variables in longitudinal studies. Such studies are under way in our laboratory.

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