

Increased Proportions of Bacteria Capable of Cleaving IgA1 in the Pharynx of Infants with Atopic Disease

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ABSTRACT

Based on the observation that children with a history of atopic disease show significantly increased levels of cleaved secretory IgA in nasopharyngeal secretions, we have previously formulated the hypothesis that bacteria-induced local deficiencies of the immune barrier of the upper respiratory tract may be a contributing factor in the development and perpetuation of atopic diseases. To evaluate this hypothesis, 25 infants were subjected to clinical, bacteriologic, and immunologic examination at the age of 18 mo, 30 mo, and 5 y. The 11 infants, who showed clinical and immunologic evidence of atopic disease at the age of 18 mo, harbored significantly higher proportions of IgA1 protease-producing bacteria (median, 36%; range, 14–64%) than the 14 healthy infants (median, 5%; range, 0.4–14%). No statistically significant differences were observed at the two subsequent examinations, but healthy children showed a statistically significant increase in proportions of IgA1 protease-producing bacteria in the pharynx with increasing age. IgA1 protease-

producing bacteria detected included *Streptococcus mitis* biovar 1, *Haemophilus influenzae*, *Haemophilus parahaemolyticus*, *Streptococcus pneumoniae*, and *Neisseria meningitidis*, of which the first mentioned species was mainly responsible for the difference observed at the 18-mo examination. Percentage proportions of IgA1 protease-producing bacteria were significantly related to passive smoking which may stimulate the premature and more pronounced pharyngeal colonization of the atopic infants with the IgA1 protease-producing variant of *S. mitis* biovar 1. The results of the study support the hypothesis that IgA1 protease-producing bacteria colonizing the upper respiratory tract jeopardize the local immune barrier and, thereby, may facilitate the penetration of potential allergens resulting in atopic disease. (*Pediatr Res* 38: 182–186, 1995)

Abbreviation

S-IgA, secretory immunoglobulin A

The mucosal membranes of the upper respiratory tract are among the most important surfaces through which sensitizing allergens pass. The principal mediator of specific humoral immunity at mucosal membranes of the body, including the upper respiratory tract, is S-IgA. Besides protecting the surfaces against infectious agents, S-IgA is capable of inhibiting the penetration of macromolecular antigens including potential allergens across mucosal membranes (1, 2). Clinical evidence for this function includes the demonstrated association of atopic disease with permanent or transient IgA deficiency (3–6).

S-IgA in the upper respiratory tract consists almost exclusively of the IgA1 subclass (7) which is susceptible to bacterial IgA1 proteases that specifically cleave the heavy chain of IgA1

in its hinge region. IgA1 proteases are secreted by several bacterial species that reside in the upper respiratory tract such as *Haemophilus influenzae*, *Haemophilus parahaemolyticus*, *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Streptococcus sanguis*, *Streptococcus mitis* (biovar 1), and *Streptococcus oralis* (8–12). We have previously examined the degree of IgA1 protease-induced cleavage of S-IgA in nasopharyngeal secretions of a group of children. Among 97 children (age 2.6–5.7 y) characteristic cleavage fragments of S-IgA1, *i.e.* monomeric Fab fragments and dimeric Fc fragments associated with the secretory component (Fc₂-SC), were demonstrated at a significantly higher prevalence (61.5% versus 11.9%) in secretions from atopic children than in healthy controls (13, 14). On this basis we presented the hypothesis that a local functional deficiency in the immune barrier of the upper respiratory tract due to extensive bacteria-induced cleavage of S-IgA1 may be a factor contributing to the development and/or perpetuation of certain atopic diseases.

This report describes a longitudinal clinical, bacteriologic, and immunologic study of a group of children from birth to 5 y of age designed to evaluate this hypothesis.

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METHODS

Study population. The study included 25 infants who were part of a population study of cord blood IgE and the development of atopic disease (15). From a birth cohort of 1625 infants born at the University Hospital of Odense, Denmark, 196 infants had a cord blood IgE concentration of ≥ 0.5 kU/L. These 196 infants were followed clinically together with 242 control infants with IgE levels within the normal range (≤ 0.3 kU/L). The 25 infants included in this study were the first of each of the two groups seen in the morning at the out-patient clinic at the 18-mo examination. Twelve of the infants had a family history of atopic disease (one or both parents having atopic disease) and a cord blood IgE level of ≥ 0.5 kU/L. The remaining 13 infants had cord blood IgE levels of ≤ 0.3 kU/L and no family history of atopic disease. All 25 infants were followed for 18 mo, 23 of these for a further 12 mo, and 20 for a total of 5 y. Informed consent was obtained from parents of the infants included in the study, and the study protocol was approved by the regional Ethics Committee for Scientific Research.

Clinical examination. At the age of 18 mo, 30 mo, and 5 y the children visited the out-patient clinic. They underwent a physical examination, and a complete history was taken with emphasis on atopic disposition, diet, housing, pets, smoking habits of the parents, atopy in the family, and infections. Atopic disease was defined as one or more of the following clinical symptoms: 1) bronchial asthma; 2) atopic dermatitis (areas of scaly, erythematous, and itchy eczematous rash with atopic localization); 3) two or more episodes of urticaria of known origin; and 4) two or more episodes of gastrointestinal allergy or intolerance to specific foods with at least one, objectively verified, positive food challenge. These criteria are identical to those used by van Asperen *et al.* (5) and Croner *et al.* (16) except that, relative to the former (5), nonimmediate food reactions were included in our study.

Immunologic examination. Blood samples for IgE determination and radioallergosorbent test for several allergens were taken if the parents permitted this at the age of 18 mo and 5 y.

Bacteriologic examinations. A throat swab collected with a coal-impregnated cotton swab from the posterior oropharyngeal wall of each child was transported to the bacteriologic laboratory in modified Stuart's transport medium (Statens Seruminstitut, Copenhagen, Denmark). Within 24 h of collection the swab was transferred to 3 mL of cold brain heart infusion broth (Difco, Detroit, MI), and the sample was suspended using a Whirly mixer for 10 s. Aliquots of 0.1 mL of appropriate 10-fold dilutions in cold brain heart infusion broth were spread with a sterile bent glass rod on duplicate sets of blood agar and on heated blood (chocolate) agar plates containing 300 mg of bacitracin/L. The inoculated agar plates were incubated for 2 d in an atmosphere of air plus 5% carbon dioxide.

The total number of colony forming units in the sample was calculated on the basis of colonies counted on agar plates with from 30 to 300 colonies and taking the dilution factor into account. The relative proportion of *Haemophilus* species in each sample was calculated on the basis of the number of *Haemophilus* colonies growing on the selective chocolate agar relative to the

total number of colonies growing on the nonselective blood agar taking the respective dilution factors into account.

From each of one selected blood agar and chocolate agar plate culture of each sample 50 colonies were subcultivated to purity, and each culture was examined for IgA1 protease activity by immunoelectrophoresis, using purified myeloma IgA1 as substrate as described previously (10). Isolates showing IgA1 protease activity were finally identified using standard techniques (17–19).

Statistical analysis. Comparison between groups was performed with nonparametrical, two-tailed Mann-Whitney *U* test and comparisons between several groups with the Friedman test. Fischer's exact test was used for statistical evaluation of 4-fold tables, and the Spearman test was used for correlation analyses.

RESULTS

Clinical results. Of the 25 infants examined, 11 satisfied the clinical and immunologic criteria for having atopic disease at the 18-mo examination. Several of the 11 infants showed multiple clinical signs of atopy (Table 1). Among the 11 atopic infants six had shown significantly raised levels of IgE (≥ 0.5 kU/L) in cord blood. The remaining five atopic infants showed IgE levels in cord blood of < 0.1 – 0.1 kU/L (Table 1). Two of the atopic infants (subjects 4 and 5) experienced recurrent gastrointestinal and upper respiratory tract infections, respectively, in addition to signs of atopy. One of the nonallergic infants (subject 15) suffered from recurrent middle ear infections. Atopic disease at the 18-mo examination was significantly correlated with passive smoking ($p = 0.0051$; 4-fold table).

At the 30-mo examination eight of the 23 children examined showed clinical signs of atopic disease. Two of the eight children with atopy at the 30-mo examination (subjects 20 and 24) had shown increased IgE levels in cord blood (1.3 and 1.4 kU/L) but revealed no clinical sign of atopic disease at the 18-mo examination. At the 5-y examination only three of the 20 children examined fulfilled the criteria for having atopic disease.

Bacteriologic findings. The total bacterial counts in the samples calculated on the basis of colonies growing on the nonselective blood agar were in the range of approximately 10^5 to 5×10^6 . The corresponding *Haemophilus* counts, including all *Haemophilus* species, calculated on the basis of growth on the selective chocolate agar, ranged from undetected (no growth from undiluted sample suspension) to 3×10^6 . *H. influenzae* constituted from 0 to 23% (median, $< 1\%$) of the bacterial flora at the 18-mo examination, from 0 to 95% (median, 1.7%) at the 30-mo examination, and from 0 to 63% (median, $< 1\%$) at the 5-y examination.

IgA1 protease activity and atopic disease. Approximately 5 500 bacterial isolates were examined for IgA1 protease activity. The relative proportion of the cultivable oropharyngeal bacterial flora in each subject at each of the three examinations that possessed IgA1 protease activity is shown in Table 1. In infants 18 mo of age without signs of atopic disease a median proportion of 5% (range, 0.4–14.0%) of the nasopharyngeal bacteria showed IgA1 protease activity. The proportions were significantly higher ($p < 0.0005$) in infants with atopic disease (median, 36%; range, 14–64%) (Fig. 1). The two children who

Table 1. Clinical and immunologic findings and the proportion of IgA1 protease-producing bacteria in the pharynx of 25 children examined at the age of 18 mo, 30 mo, and 5 y, grouped according to the presence of atopy at the first examination

Child	IgE in cord blood*	Passive smoking	Serum IgE*	18 mo		30 mo		5 y	
				Atopic disease†	% IgA1 P‡	Atopic disease	% IgA1 P	Atopic disease	% IgA1 P
1	4.20	+	774.0	CMA	30	Excema	46	None	80
2	<0.1	+	ND¶	U	14	None	20	None	20
3	2.4	+	51.0	AD, AB, CMA	38	U, CMA	11	Asthma	48
4	0.8	+	1250.0	AD, AB	64	AD, AB	33	AD	12
5	<0.1	+	8.0	AD	38	None	12	None	68
6	<0.1	+	6.0	AD	18	AD	2	None	40
7	0.8	+	ND	AD, U	52	AD, U	12		
8	0.1	-	16.0	AD	31	AD	64	None	36
9	<0.1	+	8.0	AB	22	None	10		
10	0.6	+	36.0	U, AB	36	None	10	U	88
11	0.6	+	ND	AB	58	None	2	None	68
12	<0.1	+	11.0	None	10			None	8
13	<0.1	-	6.0	None	2	None	62	None	60
14	<0.1	-	ND	None	0.4	None	23	None	40
15	<0.1	+	2.6	None	14	None	0	None	64
16	<0.1	-	9.0	None	0.8	None	6	None	80
17	3.0	-	24.0	None	12	None	23	None	36
18	<0.1	-	7.0	None	2	None	98		
19	1.6	-	4.2	None	6	None	64		
20	1.3	-	30.0	None	4	AD, AB	10	None	12
21	0.8	-	7.0	None	2	None	62	None	48
22	<0.1	-	2.0	None	10	None	10		
23	0.3	+	4.2	None	12			None	68
24	1.4	+	ND	None	2	AB	32	None	44
25	0.6	-	ND	None	14	None	95	None	100

* IgE levels expressed as kU/L.

† Abbreviations: CMA, cows milk allergy; AD, atopic dermatitis; AB, asthmatic bronchitis; U, urticaria. Data are lacking for children who dropped out of this study.

‡ IgA1 protease-producing bacteria; percentage proportion of total cultivable bacteria.

¶ ND, not done because of missing samples.

developed atopy between the first and second examination and had increased IgE levels in cord blood showed low levels of IgA1-producing bacteria at the 18-mo examination. At the 30-mo examination the proportion of IgA1 protease-producing bacteria in the pharyngeal flora of the eight children with atopy (median, 22%; range, 2–64%) was similar to those found in the 15 nonatopic children (median, 20%; range, 0–95%). At the 5-y examination only three children were atopic. Consequently, no statistical analysis of the data from the 5-y examination was performed. A comparison of the figures for the healthy children at 18 mo, 30 mo, and 5 y of age revealed a statistically significant increase ($p = 0.00166$) in the proportion of pharyngeal bacteria that had IgA1 protease activity with increasing age. The same pattern was not observed in the atopic children who had high initial levels of IgA1 protease-producing bacteria.

Analysis of the of the IgE levels in cord blood and proportions of IgA1 protease-producing bacteria at the 18-mo examination by the Spearman test revealed no significant correlation between the two parameters.

All isolates with IgA1 protease activity were subsequently identified to species level. Table 2 summarizes the findings for the 18-mo examination. *S. mitis* biovar 1 constituted the majority of the bacteria with IgA1 protease activity. Only in three children (subjects 8, 9, and 11) with atopic disease did *H. influenzae* and *N. meningitidis* contribute significantly to the

group of IgA1 protease producers observed during the study. Among the 15 children who were found to be nonatopic at the 30-mo examination, five carried high levels of IgA1 protease-producing bacteria (62–95% of the flora), whereas the remaining showed relatively low levels (median 10%; range 0–23%). In three of the children with high levels, *H. influenzae* accounted for the majority of the observed protease producers (98 out of 98%, 95 out of 95%, and 55 out of 62%). In contrast, in the children with low proportions of IgA1 protease-producers, *S. mitis* biovar 1 predominated, whereas *H. influenzae* constituted a minor part of the flora (0–17%). Other IgA1 protease-producing species were found in insignificant numbers (data not shown).

IgA1 protease activity and passive smoking. Fifteen of the 25 infants included in the study were exposed to passive smoking in their respective homes (Table 1). There was a strong association between passive smoking and increased levels of IgA1 protease-producing bacteria in the pharyngeal microflora at the 18-mo examination ($p = 0.0028$). The median proportions of IgA1 protease-producing bacteria in the pharynx of the passive smokers and nonsmokers were 26% (range 2–64%) and 4% (range 0.4–31%), respectively.

DISCUSSION

The concept that development of atopic disease may be associated with IgA deficiency is based on three lines of

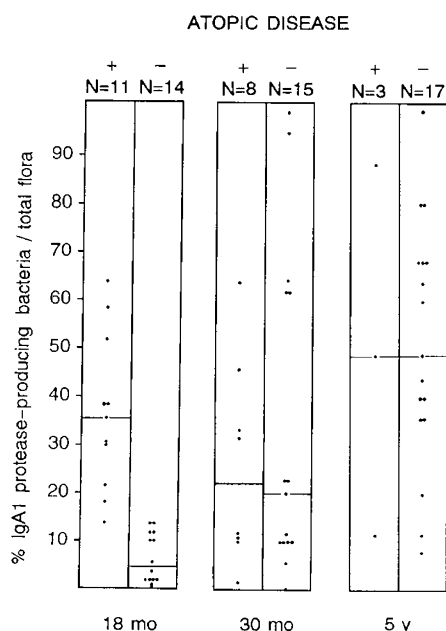


Figure 1. Percentage proportions of IgA1 protease-producing bacteria in the pharyngeal bacterial flora of a group of children examined at the age of 18 mo, 30 mo, and 5 y as related to clinical and immunologic signs of atopic disease. Horizontal lines represent median values.

evidence. First, IgA deficiency results in increased penetration of intact macromolecules through mucosal membranes (1, 21). Second, an increased prevalence of IgA deficiency or reduced levels of S-IgA in nasal secretions has been observed in atopic subjects (3, 6), and third, comparatively low levels, yet within normal range, of serum IgA was found in atopic infants (4, 20). In line with these observations van Asperen *et al.* (5) found an increased prevalence of reduced levels or absence of salivary IgA in infants up to 20 mo of age and born to atopic parents compared with controls. Studies in older children (5, 22, 23) and adults (24) have failed to demonstrate differences in S-IgA levels between atopic and nonatopic subjects.

Collectively, these studies suggest that the determining difference with regard to lack of or reduced IgA-mediated mucosal protection against sensitization occurs early in life. Our demonstration of significantly increased proportions of IgA1 protease-producing bacteria in the oropharynx of atopic children at the 18-mo examination compared with healthy controls, but not at later examinations, is in agreement with this notion. These bacteriologic results corroborate our previous demonstration of a significantly increased prevalence of extensive cleavage of S-IgA in nasopharyngeal secretions of atopic children resulting in virtual absence of intact, functionally competent IgA molecules and, thus, in an impaired immune

barrier (13, 14). Although the ensuing break in the immune barrier is restricted to the upper respiratory tract, the facilitated penetration of potential allergens may conceivably result in hypersensitivity reactions at distant sites such as the gastrointestinal canal and the skin, which is in agreement with the variety of clinical signs of atopy observed in this study. However, it cannot be excluded that IgA1 proteases of bacteria swallowed from the upper respiratory tract exert a direct effect on IgA1 in the gastrointestinal canal.

An important question is whether the presence of IgA1 protease-producing bacteria and the ensuing local deficiency of functional S-IgA is a contributing cause of the development and perpetuation of the atopic condition in the infants or merely is a secondary event. Increased occurrence of *Staphylococcus aureus* and β -hemolytic streptococci on the skin of atopic dermatitis patients has been noted (25), and an association between development of atopy and respiratory virus infection has been suggested (26, 27). Theoretically, an increased prevalence of potential pathogens such as *H. influenzae*, *S. pneumoniae*, and *N. meningitidis*, which produce IgA1 protease, might reflect an increased infection susceptibility associated with but not causing the atopic condition. However, none of these potential respiratory pathogens, which produce IgA1 protease, was found at higher prevalences in the atopic children. Surprisingly, the IgA1 protease-producing species that accounted for the significant difference between the two groups was *S. mitis* biovar 1 which occurs in a protease-producing and a nonproducing variety (12, 19). Both are commensals of the oral cavity from infancy and, to a lesser extent, the oropharynx of adults (28, 29), and there is no reason to suspect that either is a respiratory pathogen.

Rather than reflecting a lifelong difference in the ability to become colonized by the IgA1 protease-producing variety of *S. mitis* biovar 1, our data indicate a premature predominance of this species in the pharynx of infants developing atopic disease. The reason for this unexpected finding is unknown. Passive smoking may be a contributing factor. Our data revealed a strong association between passive smoking and increased proportions of IgA1 protease-producing bacteria. Although smoking merely may be a confounding factor, it has been previously shown that tobacco smoke components may have a selective toxic effect on particular species inhabiting the respiratory tract (30, 31). *In vitro* studies have indicated that *S. mitis* and other viridans streptococci are less susceptible to the harmful effects of cigarette smoke than several Gram-negative species (32). Clinically, several studies have demonstrated an association between passive tobacco smoking and development of asthma and atopic disease (33–35).

Table 2. Proportions and identity of IgA1 protease-producing bacteria in the pharynx of infants with and without signs of atopic disease at the age of 18 mo

Clinical status	No.	Median % of total bacterial flora (range)					<i>S. pneumoniae</i>
		All protease-producing species	<i>S. mitis</i>	<i>H. influenzae</i>	<i>N. meningitidis</i>	<i>H. parahaemolyticus</i>	
Atopy	11	36 (14–64)	30 (0–63)	1 (0–23)	0 (0–57)	0 (0–2)	0
No atopy	14	5 (1–14)	5 (0–12)	<1 (0–2)	0 (0–4)	0 (0–2)	0 (0–2)

The activity of bacterial IgA1 proteases are inhibitable by antibodies induced in the host. A given *H. influenzae*, pneumococcal, or meningococcal IgA1 protease may be anticipated to result in IgA cleavage only for a short period of time. Thus, clonal types of noncapsulated *H. influenzae*, which express a significant diversity of antigenic forms of IgA1 proteases, are being rapidly cleared or replaced concurrent with the development of antibodies by the host (36). The extremely high proportion of IgA1 producing *H. influenzae* in three nonatopic children at the 30-mo examination is likely to be a temporary situation possibly associated with an upper respiratory tract or middle ear infection. In contrast to *H. influenzae*, *S. pneumoniae*, and *N. meningitidis* IgA1 proteases (36, 37), enzymes produced by viridans streptococci lack antigenic variation (12) in spite of the fact that the species are found as members of the upper respiratory tract flora throughout life. Therefore, the IgA1 proteases of viridans streptococci are likely to be active only as long as neutralizing antibodies are lacking, *i.e.* in the first part of life. Antibodies to IgA1 proteases of viridans streptococci do not regularly develop as a result of mere colonization (38, 39) and may be induced primarily by cross-reactive proteases released by concurrently colonizing *S. pneumoniae* (39, 40). Consequently, the *S. mitis* IgA1 protease conceivably contributes to impairment of the mucosal barrier mainly during the first years of life and is less relevant in older children, in whom high levels of this species were found, and also in children without atopic disease (Fig. 1). Antibody-induced inhibition of IgA1 proteases may, thus, lead to an eventual restoration of an intact mucosal barrier and to eventual recovery from the atopic condition as indicated also by the significant reduction of the proportion of children with atopy during the 5-y observation period of this study.

Although a causal relationship has not been established, the results of this and our previous study (13, 14) suggest that IgA1 proteases secreted by some of the bacteria colonizing the pharynx of infants induce deficiencies of the local immune barrier of the upper respiratory tract and/or increased local hyperreactivity. A premature pronounced colonization, possibly stimulated by passive smoking, of the IgA1 protease-producing variant of *S. mitis* appears to be particularly responsible. This bacteria-induced functional deficiency of the immune barrier may lead to allergic sensitization in otherwise predisposed infants.

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