

## Comparison of Agglutinin Titers for *Streptococcus mutans* in Tears, Saliva, and Serum

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The agglutinin titers for three *Streptococcus mutans* serotypes (AHT, BHT, and 10449, representing serotypes *a*, *b*, and *c*, respectively) were measured in the saliva, tears, and serum of 19 human subjects. Naturally occurring *S. mutans* agglutinins were routinely present in all fluids tested in the absence of overt local stimulation by antigen. The immunoglobulin A nature of this secretory agglutinin activity was suggested by blocking with alpha heavy-chain-specific antiserum and by the demonstration of *S. mutans*-reactive immunoglobulin A in the saliva and tears by indirect immunofluorescence. This finding is consistent with stimulation and antigen commitment of immunoglobulin A precursor lymphocytes at remote sites and subsequent homing to the lacrimal system. The relationship of anti-AHT agglutinins to anti-10449 agglutinins differed among the body fluids tested. The tears had more agglutinins for strain AHT than for strain 10449, whereas the reverse was true for saliva and serum. A possible explanation is local antigen-driven expansion of AHT-reactive committed lymphocytes in the lacrimal tissues.

Evidence that secretory immunoglobulin A (sIgA) is the major immunoglobulin in secretions (27, 28) suggests its importance in defending mucosal surfaces. A number of investigators have shown that sIgA prevents adherence of bacteria to mucosal surfaces and functions in the disposal of bacterial antigens (8, 26, 29). Immunoglobulin A (IgA) in secretions such as tears may help prevent bacterial colonization on the ocular surface (7).

Studies by Mestecky et al. (18) and McGhee et al. (16) of orally ingested *Streptococcus mutans* in humans have demonstrated the simultaneous appearance of anti-*S. mutans* sIgA antibodies in saliva and tears. Montgomery et al. (22), using rabbits, found that both bronchial and gastric routes stimulate sIgA antibodies in peripheral secretions such as milk and saliva, as well as in bronchial and intestinal fluid. Other animal studies have indicated that Peyer's patch cells, when stimulated by antigen, home to remote exocrine sites where they produce sIgA (20, 23) or promote resident lymphocytes to produce it (12, 13, 24). These studies and the fact that naturally induced antibodies to antigen appear in secretions in sites where antigen stimulation could not occur (1) suggest the existence of a common mucosal system that does not require endogenous antigen for antibody production.

The absence of antibody in serum in experiments of Mestecky et al. (18) and McGhee et al.

(16) also suggests that there is selective local stimulation of antibody at the salivary and lacrimal sites and no measurable stimulation of the spleen-lymph node system. Local presentation of antigen has been found to stimulate secretory antibodies at the site of immunization (9, 17). Assuming that a common mucosal system (2, 15) exists, it is probable that local stimulation involves expansion of previously committed cells rather than the alternative of local stimulation of uncommitted cells. Thus, it was of interest to determine whether natural antibodies to nonocular antigen are present in tears.

In previous studies of exocrine secretions, the predominant antibody type corresponded to the predominant bacterial types present (4, 25). The profiles of *S. mutans* antibody specificity for colostrum, serum, and saliva were similar in that all had higher levels of antibody to *S. mutans* serotype *c* than to *S. mutans* serotype *b* (1).

This study examines the agglutinin activity of tears, saliva, and serum to *S. mutans* serotype *a* (strain AHT), type *b* (strain BHT), and type *c* (strain 10449).

### MATERIALS AND METHODS

**Subjects.** Nineteen subjects with no evidence of ocular inflammation, dilated blood vessels, exudates, or mucus, who ranged in age from 19 to 55 years, were randomly selected for study. Tears, saliva, and serum were collected from each subject.

**Collection of samples.** At least 100  $\mu$ l of tears was collected on cellulose sponges (Weck-Cell) placed in the inner canthus. Tear flow was not stimulated in any other way. The sponges were then placed in 0.3-ml plastic centrifuge tubes, each having a small hole at the tip. Centrifugation transferred the tears from the sponges into a second tube from which the fluid was removed for assay.

At least 1 ml of parotid saliva was collected by an intraoral cup (1) and was then centrifuged to remove cells and particulate debris.

Venous blood (10 ml) was drawn and allowed to clot. Serum was removed and stored until agglutination was performed. Samples were not frozen before assay.

**Agglutinin titers.** Agglutinin titers were determined with a microtiter system (Dynatech Laboratories, Inc., Alexandria, Va.). Barbitol buffer (pH 7.2) containing 1.1% bovine serum albumin was used for twofold dilution of 50- $\mu$ l volumes of each sample of saliva and serum and most tear samples. Antigen was suspended to  $2 \times 10^8$  colony-forming units (determined by absorbance at 660 nm of approximately 0.1) of Formalin-killed *S. mutans* in barbitol-bovine serum albumin buffer, and 50- $\mu$ l volumes were added to each well of a V-bottom plate. The plates were incubated at 37°C for 2 h and at 4°C overnight. Agglutinin titers were read with the aid of a dissection microscope.

The endpoint of agglutination was taken as the last well to give a pattern different from that of the buffer control. The sensitivity of the technique was  $\pm 1$  well. Tear samples were run in duplicate (owing to limited volumes); saliva and serum samples were run in triplicate. The limited volume of saliva and tears prevented the determination of titers for all bacterial strains for each sample. Selected saliva and tear samples were incubated with an optimal concentration of goat anti-human alpha heavy-chain serum at 37°C for 3 h before agglutinin titration (18).

***S. mutans* strains.** The strains of *S. mutans* used were AHT (Bratthall serotype a), BHT (Bratthall serotype b), and 10449 (Bratthall serotype c). All strains were reconstituted from lyophilized stock and grown at 37°C in a partially defined medium (5). Overnight cultures were harvested by centrifugation, washed three times in sterile saline, and suspended in 0.5% formalinized saline. After 72 h at room temperature, the cells were washed and suspended in sterile saline and checked for sterility. For microtitration, the cells were resuspended at  $2 \times 10^8$  colony-forming units per ml. The means of identifying the various *S. mutans* strains have been described previously (3).

**Indirect immunofluorescence.** Heat-fixed smears of 18-h cultures of the various *S. mutans* strains were incubated for 45 min at ambient temperature with

selected samples of parotid saliva and tears. After being thoroughly washed in phosphate-buffered saline (pH 7.5), the smears were incubated with fluorescein isothiocyanate-labeled anti-alpha chain antiserum (Behring Diagnostics, Somerville, N.J.) which did not contain any antibodies to *S. mutans*. After a second washing, the smears were observed with a Leitz Ortholux fluorescence microscope equipped with vertical Ploem illumination and filters for narrow-band excitation.

**Statistics.** The antibody titers were converted to  $\log_2$  values. Ratios of the titer of one strain to that of another strain were made for each of the three fluids for each subject (e.g., the ratio of the titer of AHT/10449 for the tears of subject 1 was 4/11; the ratio of AHT/10449 for the saliva of the same subject was 1/2). The ratios of each strain pair in one fluid were compared with the ratios of the same strain pair in the other fluids with the paired *t* test (11). A *P* value of less than 0.05 was considered significant in determining the prevalence of AHT/10449 antibody titers  $\geq 1$  for tears as compared with saliva. The McNemar test (6) for correlated proportions was used. The paired *t* test was used to compare the ratio of two antibody levels in one fluid with the ratio of the same two antibody levels in a second fluid (11).

## RESULTS

All samples tested had agglutinin activity for each of the three serotypes of *S. mutans* (Table 1). The IgA nature of this agglutinin activity was suggested by indirect immunofluorescence and agglutinin blocking with alpha chain-specific antiserum. The agglutinin titers for strains AHT and 10449 were higher for tears than for saliva, which in turn were higher than those for serum. Mean titers for BHT were considerably lower in all three fluids than were titers for AHT or 10449. The agglutinin titers for the three serotypes differed in tears, saliva, and serum for the same subjects (Table 2).

In tears, agglutinins for AHT exceeded those for 10449, which in turn exceeded those for BHT. Tears sufficient for all agglutinins were available from 9 of the 19 subjects (Table 1). Values from these nine were used in the paired *t* test for tears (Table 2). In saliva, agglutinins for 10449 were greater than those for AHT, which were greater than those for BHT. These comparisons were made on saliva samples from 18 subjects. In serum, agglutinins for 10449, BHT, and AHT were equivalent in comparisons made on all 19 subjects.

TABLE 1. Average titers<sup>a</sup> of anti-*S. mutans* antibody in tears, saliva, and serum

Body fluid	AHT			BHT			10449		
	No.	$\bar{x}^b$	$\pm 1$ SD	No.	$\bar{x}$	$\pm 1$ SD	No.	$\bar{x}$	$\pm 1$ SD
Tears	17	1,024	512-2,048	17	32	8-128	9	512	128-2,048
Saliva	18	128	32-512	18	32	8-128	18	256	128-512
Serum	19	32	16-64	19	32	16-64	19	128	64-256

<sup>a</sup> Performed on  $\log_2$  values.

<sup>b</sup>  $\bar{x}$ , Mean.

TABLE 2. Comparison<sup>a</sup> of mean ratios of anti-*S. mutans* antibody titers in tears, saliva, and serum<sup>b</sup>

Body fluid	AHT/10449			AHT/BHT			10449/BHT		
	$\bar{x}^c$	<i>t</i>	<i>P</i>	$\bar{x}$	<i>t</i>	<i>P</i>	$\bar{x}$	<i>t</i>	<i>P</i>
Tears	1,024/512	2.83	<0.05	1,024/32	12.95	<0.001	512/32	9.54	<0.001
Saliva	128/256	-2.44	<0.05	128/32	6.86	<0.001	256/32	12.54	<0.001
Serum	32/128	-1.835	NS <sup>d</sup>	32/32	-1.11	NS	128/32	0.56	NS

<sup>a</sup> Paired *t* test.

<sup>b</sup> Nine sets of tears, saliva, and serum.

<sup>c</sup>  $\bar{x}$ , Mean.

<sup>d</sup> NS, Not significant.

A surprising observation was the relationship of AHT to 10449 agglutinins among the three fluids. Both saliva and serum infrequently had higher levels of agglutinins for AHT as compared with those for 10449. For saliva, 5 of 18 AHT/10449 ratios were greater than 1; for serum, 5 of 19 ratios were greater than 1. Tears, however, had a consistently higher level of AHT/10449 agglutinins in the same person (nine of nine tear samples had AHT/10449 ratios  $\geq 1$ ). The tear ratios differ from the saliva ratios at the 0.02 level (McNemar test).

### DISCUSSION

Tears contain natural agglutinins for the oral streptococcus *S. mutans*. Previous studies have shown that this agglutinin activity in colostrum and saliva is due to sIgA antibody (16, 18). Ingestion of streptococci, stimulation of the gut, and the subsequent homing to the mammary gland and parotid salivary gland have been the proposed mechanism (17, 19). Although secretory antibody is found at both of these sites, these glands are remote from the site of bacterial growth (2, 10). Since the normal ocular flora does not contain *S. mutans*, ingested *S. mutans* antigens may likewise be responsible for the expression of natural antibodies of the IgA class in tears (18).

The level of agglutinin activity for *S. mutans* appears to be related to the predominant serotype present in the oral cavity. We have found that the relationship of the levels of anti-AHT to anti-10449 agglutinins is similar for saliva, colostrum, and serum (1). In addition, others have found that *S. mutans* serotype *c* is the dominant oral type (4, 25) and that agglutinins for serotype *c* dominate in saliva, colostrum, and serum. Since the parotid and mammary glands are remote from the environment, local stimulation of antibody-forming precursor cells is unlikely, which suggests that remote stimulation probably occurs.

In this study, however, the ratio of anti-AHT agglutinin levels to anti-10449 agglutinin levels in tears, unlike the ratios for saliva and serum, was consistently greater than 1. Since the toni-

ty of saliva is less than that of tears (21), perhaps this difference could have influenced agglutination. Glycoprotein aggregates or protein aggregating factors (14) could also have caused increased agglutination. However, a more likely explanation for the disparity of agglutinin levels for the tears as compared with those for saliva is local influence in the lacrimal system (if cells distributed to the salivary and lacrimal area are indeed of common origin). Bacterial antigens on the ocular surface may have access to lymphocytes in or near the lacrimal system (main lacrimal gland, accessory glands, and possibly conjunctiva), and these antigens may influence the expansion of previously committed cells. It is possible that among the normal flora of the eye are organisms that share antigenic determinants with *S. mutans* serotype *a* and that these bacterial antigens selectively stimulate expansion of anti-serotype *a*-committed cells.

Less likely explanations are that lymphoid cells are distributed to lacrimal and salivary systems from different sites or from one site but migrate at a different rate to the lacrimal system than to the parotid system. Alternatively, the different ratios for tears as compared with serum and saliva may represent either the encouragement of agglutinin production in lacrimal tissue or the repression of agglutinin production in salivary and serum systems or both.

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