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Antigenic Variation of *Streptococcus mutans* Colonizing Gnotobiotic Rats

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Strains of *Streptococcus mutans* representative of serotypes *b* and *d* exhibited antigenic variation in both the oral cavity and in the intestinal canal of gnotobiotic rats. Laboratory-maintained cultures did not vary. The antigenic alterations observed were: (i) loss of detectable levels of both weakly reacting "strain" antigens and the type antigen; (ii) decreased production of the type antigen; (iii) production of altered type antigen; and (iv) production of an antigen not possessed by the parent strain. Immunization of animals before monoinfection with *S. mutans* strain Bob-1 (serotype *d*) appeared to increase the rate of emergence of antigenically altered mutants in the intestinal canal, and more diversely altered isolates were obtained. Antigenic variation may account in part for the variation noted by several investigators in attempting to immunize animals against *S. mutans*-induced dental caries.

Several infectious agents have been shown to exhibit antigenic variation during the course of colonizing a mammalian host. This phenomenon, sometimes referred to as antigenic drift, has been classically associated with influenza virus infections, and it probably occurs with other viral agents (9). Antigenic variation occurs in protozoan (6) and bacterial infections as well. In the case of bacteria, the serotypes of *Escherichia coli* inhabiting the human intestinal canal have been observed to change over time (7, 19). Similarly, populations of *Vibrio cholerae* in monoinfected gnotobiotic rats change serotype, probably because antibodies induced to certain antigenic components of the initial colonizing organisms cause the selection of antigenically altered mutants (17, 20). Populations of *Campylobacter fetus* also have been reported to exhibit antigenic variation while causing persistent venereal vibriosis in cows (8), and it has been suggested that this enables the organism to elude the host's immune response (1, 8). In streptococci, it has been shown that both group and type antigens can be lost during serial mouse passages (14, 29).

Human oral sections frequently contain detectable titers of antibodies reactive with strains of bacteria that normally inhabit the mouth (5, 13, 22, 28). In addition, many bacteria present in saliva have been found to be coated with immunoglobulin (Ig) A (3), and significant quantities of both IgA and IgG are present in the microbial deposits that collect on human teeth (24). Since antibodies, including those of salivary IgA, can inhibit the attachment of bacteria to oral surfaces (28), their presence in oral secretions should influence the colonization of affected bacteria. The agglutinating activities of salivery IgA preparations reactive against strains of oral streptococci have recently been found to change over a 150-day period, suggesting that the populations of streptococci colonizing the mouth also fluctuate over time (5). The present report describes changes in the antigenic composition of populations of *Streptococcus mutans*, an organism associated with dental caries activity, while colonizing the oral cavity and intestinal canal of gnotobiotic rats.

MATERIALS AND METHODS

Cultures and cultural conditions. *S. mutans* strain FA-1 (serotype *b*) was a rat isolate kindly provided by R. J. Fitzgerald. *S. mutans* strain Bob-1 (serotype *d*) was freshly isolated from human dental plaque (kindly provided by R. Berkowitz). The organisms were maintained by weekly transfer in Trypticase soy broth (BBL) and on mitis-salivarius agar plates (Difco).

Experimental procedures. A group of four germ-free Sprague-Dawley rats, 26 days old, was maintained in flexible plastic isolators as previously described (10) and fed diet 2000 (12). The animals were infected with *S. mutans* strain FA-1, and oral swabbings and fresh fecal pellets were collected from each animal periodically up to 94 days after infection. The samples were streaked on mitis-salivarius agar plates, which were incubated in Brewer jars filled with 10% CO_2, 10% H_2, and 80% N_2 at 35 C. Random colonies were picked from plates of each sample; these were restreaked to obtain pure cul-

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tures. Antigen extracts of each isolate were assayed by immunodiffusion for the presence of the b and n antigens previously described in the parent strain of FA-1 (4). Control antigen extracts were also prepared from 40 isolates derived from the original culture used to infect the animals.

In a second experimental series, two groups of four germfree rats were housed in separate isolators. One group was immunized with 0.1 ml of a vaccine of *S. mutans* strain Bob-1 in each footpad; the injections were repeated 1 week later. The vaccine used was prepared by mixing equal quantities of a saline suspension containing 10^9 formalin-killed, Trypticase soy-grown streptococci with Freund incomplete adjuvant. Both the immunized and nontreated control animals were infected with the same culture of *S. mutans* strain Bob-1 1 week after the last injection. Oral swabbings and freshly collected fecal pellets were obtained periodically, and random isolates were obtained as described above. Antigen extracts of each isolate were analyzed for the presence of the d and s antigens of strain Bob-1 by immunodiffusion.

Serological methods. Overnight Trypticase soy broth cultures of each isolate were used for preparation of the antigen extracts. The streptococci were harvested by centrifugation, washed, and suspended in saline. Each suspension was adjusted on the basis of optical density to contain 3.0×10^8 to 3.5×10^8 streptococci/ml. A 40-ml sample of each suspension was used for antigen extraction according to the method described by Rantz and Randall (18). All extracts for a given sampling period were made and autoclaved simultaneously to minimize variation in their preparation.

Rabbit antiserum was prepared by using heat-killed cells of *S. mutans* according to an immunization schedule described previously (4). Cells used for immunization were grown in a sucrose-free, predialyzed Trypticase yeast extract medium. Antiserum to *S. mutans* strain FA-1 was used to analyze antigen extracts of isolates derived from animals infected with this organism, whereas an antiserum prepared to *S. mutans* strain B13 (serotype *d*) was used to analyze isolates obtained from animals infected with strain Bob-1.

Immunodiffusion was carried out in 1.2% agar gels, using the microplate technique described by Wadsworth (26) for the FA-1 antigens or the Gelman instrument (Ann Arbor, Mich.) gel diffusion apparatus for antigens of Bob-1 isolates.

RESULTS

Antigenic variation in populations of S. mutans strain FA-1 (serotype b). The anti-FA-1 serum uniformly produced two distinct precipitin bands with antigen extracts of all 40 isolates derived from the culture used for infecting the rats (Fig. 1). The dense band represents the b type antigen and the weak band represents the n antigen as previously described (4). All isolates obtained from rats 5 to 10 days after monoinfection with *S. mutans* strain FA-1 contained b antigen, but the n antigen was not detected in the extract of 1 of 11 fecal isolates (Table 1). The percentage of isolates lacking detectable n antigen increased over time, and 94 days after initial infection, 61% of the fecal isolates and 21% of the oral isolates no longer possessed detectable levels of n antigen under the standardized conditions of extraction used (Table 1; Fig. 2). In addition, one oral isolate that no longer possessed detectable levels of either the b or n antigen was found on day 62 (Fig. 2). Other isolates altered with respect to the b antigen were cultured from additional animals at subsequent sampling periods (Table 1). By the end of the experimental period, isolates altered in either the n or b antigen were isolated from every animal studied (Table 1).

Electron microscopic examination (kindly performed by Z. Skobe, Forsyth Dental Center) of strains no longer producing detectable levels of either the b or n antigen, or both, did not reveal differences in their cell surface morphology that could be correlated with the antigenic changes.

Antigenic variation in populations of S. mutans strain Bob-1 (serotype d). Antiserum to *S. mutans* strain B13 (type *d*) produced a dense precipitin band corresponding to the d antigen and a weaker band, designated s antigen, with extracts derived from isolates from the parent strain Bob-1 culture (Fig. 3). Isolates from untreated animals were similarly homogeneous with respect to the antigenic components monitored 4 days after infection, but isolates that no longer exhibited detectable levels of s antigen were obtained after 14 days (Table 2; Fig. 4). Comparably altered isolates were present in all subsequent sampling periods, but no isolates that were altered with respect to the d antigen were obtained from these animals during the experimental period.

The populations of *S. mutans* strain Bob-1 colonizing previously immunized animals appeared to exhibit greater antigenic variation; this was particularly evident among fecal isolates (Table 3). Forty-one days after infection, 80% of the isolates no longer possessed detectable levels of s antigen, and a few oral and fecal isolates exhibited weak d antigen reactivity (Fig. 5), though none were found which totally lacked detectable levels of this antigen. Extracts of other isolates were found to produce a spur with the parent d antigen (Fig. 6), indicating another type of antigenic alteration (Table 3). At day 53, two fecal isolates produced a precipitin band with the anti-B13 serum that was not detected in extracts of the parent strain Bob-1 (Fig. 6). This antigen has been referred to as "x." At the end of the experiments, 43% of the total fecal isolates displayed this compo-

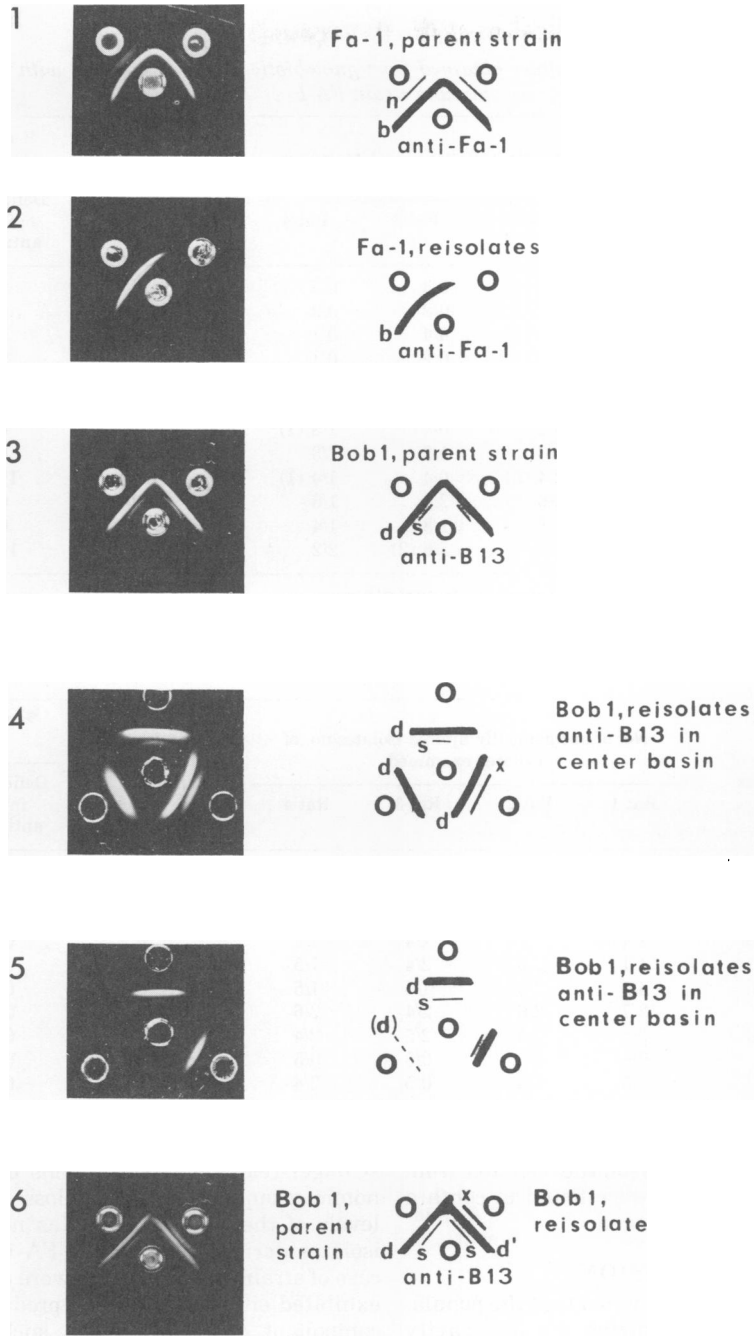

FIG. 1. *Comparative double-diffusion in gel analyses of two antigen extracts of the parent strain Fa-1, which was used for infection of the animals.*

FIG. 2. *Analyses of two antigen extracts of reisolates from rats infected with Fa-1. One isolate shows lack of detectable amounts of n antigen, the other extract of n and b antigens.*

FIG. 3. *Analyses of antigen extracts of parent strain Bob-1.*

FIG. 4. *Analyses of antigen extracts of three reisolates of strain Bob-1. One isolate shows the original composition (d+, s+), and one isolate contains the "new" antigen (d+, x+, s−). The last extract shows the d antigen only.*

FIG. 5. *Antigen extracts of reisolates of strain Bob-1 tested against anti-B13 serum. Note the weak precipitin band corresponding to the d antigen in extract in lower left basin.*

FIG. 6. *Analyses of antigen extracts of a parent strain and reisolate of Bob-1. Spur formation in the d precipitin band indicates a partial change in the type antigen of the reisolate. The reisolate also contains the s and x antigens.*

TABLE 1. *Antigenic variation of isolates obtained from gnotobiotic rats monoinfected with Streptococcus mutans strain FA-1*

Days after infection	Source of isolates	No. of antigenically altered isolates/no. of isolates examined				Total isolates altered/total isolates examined	% of isolates that were:	
		Rat 1	Rat 2	Rat 3	Rat 4		Deficient in b antigen	Deficient in n antigen
5–10	Oral	0/3	0/3	0/3	0/3	0/12	0	0
	Fecal	1/5	0/2	0/2	0/2	1/11	0	9
26–39	Oral	0/5	0/4	0/4	0/1	0/14	0	0
	Fecal	1/11	0/3	0/2	0/1	1/17	0	5
47	Oral	0/5	1/5	0/5	0/5	1/20	0	5
	Fecal	0/5	0/5	1/5	0/5	1/20	0	5
62	Oral	1/5	0/2	0/4	1/5 (1)[a]	2/16 (1)	6	13
	Fecal	0/5	0/3	1/2	1/3	2/13	0	15
80	Oral	1/4	2/4 (2)	0/4	1/4 (1)	4/16 (3)	19	25
	Fecal	1/6	2/6	2/6	1/6	6/24	0	25
94	Oral	2/4	1/8	1/8	1/4	5/24	0	21
	Fecal	3/8	1/2	5/6 (2)	2/2	11/18 (2)	11	61

[a] Isolates without detectable b antigen are in parentheses.

TABLE 2. *Antigenic variation of isolates obtained from gnotobiotic rats monoinfected with Streptococcus mutans strain Bob-1*

Day after infection	Source of isolates	No. of antigenically altered isolates/no. of isolates examined				Total isolates altered/total isolates examined	% of isolates that were:	
		Rat 1	Rat 2	Rat 3	Rat 4		Deficient in d antigen	Deficient in s antigen
4	Oral	0/5	0/5	0/5	0/5	0/20	0	0
	Fecal	0/5	0/5	0/5	0/5	0/20	0	0
14	Oral	2/5	0/4	1/4	1/5	4/18	0	22
	Fecal	2/4	1/4	1/4	1/4	5/16	0	31
41	Oral	1/1	2/5	2/4	1/5	6/15	0	40
	Fecal	4/5	2/5	1/4	1/5	8/19	0	42
53	Oral	2/7	2/6	2/4	2/6	8/23	0	34
	Fecal	2/5	2/5	2/5	2/4	8/19	0	42
74	Oral	0/5	0/5	0/5	0/5	0/20	0	0
	Fecal	0/5	1/5	0/5	2/4	3/19	0	16

nent. None of the oral isolates from the immunized animals and none of the isolates from nonimmunized animals were found to contain detectable levels of x antigen.

DISCUSSION

The present study has shown that the populations of *S. mutans* colonizing the oral cavity and the intestinal canal of gnotobiotic rats change antigenic composition. Although the antisera used to monitor antigenic changes produced only two well-defined precipitin bands against antigen extracts of the two strains studied, a variety of antigenic alterations was observed when assayed by immunodiffusion. The most frequently observed alteration was the loss of detectable levels of the weakly reacting antigen of either *S. mutans* strain FA-1 (serotype *b*) or strain Bob-1 (serotype *d*); these are probably more strain-specific antigens than the stronger-reacting type antigens used for taxonomic grouping. However, loss of detectable levels of the type antigen was noted in some isolates derived from strain FA-1, and in the case of strain Bob-1, isolates were obtained that exhibited either a decreased production of this component or an alteration such that it no longer exhibited patterns of identity with the type d antigen of the parent strain. In addition, several isolates from strain Bob-1-infected rats produced an antigenic component reactive with anti-strain B13 sera that was not evident in the antigen extract of the parent strain. It is possible that other new antigens or determinants would have been detected if antisera to additional strains had been used. The colonial morphology of several isolates differed from that of their parent strains on mitis-salivarius agar,

TABLE 3. *Antigenic variation of isolates obtained from gnotobiotic rats immunized and subsequently monoinfected with Streptococcus mutans strain Bob-1*

Day after infection	Source of isolates	No. of antigenically altered isolates/no. of isolates examined				Total isolates altered/total isolates examined	% of total isolates altered with respect to:		
		Rat 1	Rat 2	Rat 3	Rat 4		Deficiency in d antigen	Deficiency in s antigen	Production of x antigen
4	Oral	0/5	0/5	0/5	0/5	0/20	0	0	0
	Fecal	0/5	0/5	0/5	0/5	0/20	0	0	0
14	Oral	1/4	0/2	0/3	0/2	1/11	0	9	0
	Fecal	0/3	2[a]/4	1[a]/4	1/5	4[a]/16	0[a]	25	0
41	Oral	0/5	No sample	1[a]/1	1/8	2[a]/14	0[a]	14	0
	Fecal	6/6	6/9	5/6	4/5	21/26	0	80	0
53	Oral	1/5	1/4	2/7	2/5	6/21	0	28	0
	Fecal	4/4	5/5 (2)[b]	2/2	5/5	16/16 (2)	0	100	13
74	Oral	0/3	0/5	1/4	1/5	2/17	0	12	0
	Fecal	2/4 (1)	6/6 (4)	5/6 (4)	6/7 (1)	19/23 (10)	0	83	43

[a] Some isolates possessed low levels of d antigen.
[b] Isolates with x antigen are in parentheses.

but these differences did not correlate directly with the antigenic components monitored. These morphological changes probably reflect other alterations in the surface components of the *S. mutans* strains studied that were nonreactive with the antisera used. It is apparent from these observations that the antigenic composition of the populations of human and rodent strains of *S. mutans* can vary widely during the course of colonizing experimental animals.

The importance of antibodies as a mechanism for the selection of altered antigenic types has been demonstrated by Willers and Alderkamp in vitro (27). By subcultivating a nonhemolytic streptococcal strain several times in medium containing antiserum against the type antigen, they succeeded in isolating organisms lacking detectable quantities of this antigen. Studies of antigenic variation in *V. cholerae* colonizing gnotobiotic mice (17, 20) and of *C. fetus* infecting cattle (8) have suggested that the microbial population changes were mediated by an immune response to the initial colonizing populations that favored the selection of antigenically altered mutants. The observation that prior immunization increased the rate of emergence of antigenically altered mutants of *S. mutans* in the intestinal canal of monoinfected rats is consistent with this possibility; moreover, more diversely altered isolates were obtained from artificially immunized rats. The population changes observed are also consistent with the finding that the agglutinating activities of human salivary IgA preparations reactive with strains of indigenous streptococci, including *S. mutans* and *S. salivarius*, vary over time (5). In addition, strains of *S. salivarius* also exhibit antigenic variation while colonizing gnotobiotic rats (T. H. Howell and R. J. Gibbons, Abstr. Gen. Sess. Am. Assoc. Dent. Res. 1975, abstr. 628), and hence antigenic drift appears to be common among bacteria that colonize a mammalian host. Since strain FA-1 has been maintained in the laboratory for over 10 years without displaying evidence of antigenic alterations, and because isolates derived from cultures of the parent strains consistently yielded identical precipitin patterns, it is clear that the experimental animals created a selection pressure favoring the emergence of antigenically altered variants that is not present during in vitro cultivation.

S. mutans has been associated with dental caries activity in both humans and experimental animals (11, 21), and several studies in recent years have attempted to induce immunity to tooth decay by using vaccines of *S. mutans* cells. Thus, hamsters (A. Gaffar, H. W. Marcussen, J. Ruffner, and R. C. Kestenbaum, Abstr. 48th Gen. Meet. Int. Assoc. Dent. Res. 1970, abstr. 304), conventional and gnotobiotic rats (2, 16, 25), and two species of subhuman primates (2, 15) have been immunized by systemic administration of vaccines of *S. mutans*, and reductions in dental decay have varied between 0 and 60 to 80% after infection. In addition, several investigators have experienced difficulties in reproducing experiments (23, 25), and differences in protection have been noted between individual animals, particularly with primates (15). The marked variation in antigenic composition of *S. mutans* observed in the present study, especially in animals that had been systemically immunized, probably accounts for some of these variable experiences. Based upon the observations made, it seems likely that the

populations of *S. mutans* colonizing experimental animals for periods of weeks or months after immunization only partially resemble the antigenic composition of the parent strain used for vaccine production. Therefore, the probability of antigenic drift occurring in *S. mutans* populations must be considered in attempts to reduce dental caries by immunization procedures. To date, most attempts to induce immunity to dental caries have used animals that were heavily infected with *S. mutans* (2, 15, 16, 23, 25). However, it would appear that once this organism attains high population levels, the probability of antigenically altered mutants being present is also high, and hence antigenic drift likely occurs. Therefore, it appears that artificially induced immunity may be more promising in preventing initial colonization of *S. mutans* rather than in eliminating this organism after it has established an infection.

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LITERATURE CITED

1. Beale, G. H., and J. F. Wilkinson. 1961. Antigenic variation in unicellular organisms. Annu. Rev. Microbiol. 15:263–296.
2. Bowen, W. H. 1969. A vaccine against dental caries. A pilot experiment in monkeys. Br. Dent. J. 126:159–160.
3. Brandtzaeg, P., I. Fjellander, and S. T. Gjeruldsen. 1968. Adsorption of immunoglobulin A onto bacteria in vivo. J. Bacteriol. 96:242–249.
4. Bratthall, D. 1969. Immunodiffusion studies on the serological specificity of streptococci resembling *Streptococcus mutans*. Odontol. Revy 20:231–243.
5. Bratthall, D., and R. J. Gibbons. 1975. Changing agglutination activities of salivary immunoglobulin A preparations against oral streptococci. Infect. Immun. 11:603–606.
6. Brown, I. N., K. N. Brown, and L. A. Hills. 1968. Immunity to malaria: the antibody response to antigenic variation by *Plasmodium knowlesi*. Immunology 14:127–138.
7. Cooke, E. M., S. Ewins, and R. A. Shooter. 1969. Changing faecal populations of *Escherichia coli* in hospital medical patients. Br. Med. J. 4:593–601.
8. Corbeil, L. B., G. G. D. Schurig, P. J. Bier, and A. J. Winter. 1975. Bovine veneral vibriosis: antigenic variation of the bacterium during infection. Infect. Immun. 11:240–244.
9. Fenner, F., B. R. McAnslan, C. A. Mims, J. Sambrook, and D. O. White. 1974. The biology of animal viruses, 2nd ed., p. 618–640. Academic Press Inc., New York.
10. Gibbons, R. J., S. S. Socransky, and B. Kapsimalis. 1964. Establishment of human indigenous bacteria in germ-free mice. J. Bacteriol. 88:1316–1323.
11. Gibbons, R. J., and J. van Houte. 1975. Dental caries. Annu. Rev. Med. 26:121–136.
12. Keyes, P. H., and H. V. Jordan. 1964. Periodonal lesions in the Syrian hamster. III. Findings related to an infectious and transmissible component. Arch. Oral Biol. 9:377–400.
13. Kraus, F. W., and J. Konno. 1965. The salivary secretion of antibody. Ala. J. Med. Sci. 2:15–22.
14. Lancefield, R., and G. Perlmann. 1952. Preparation and properties of a protein (R antigen) occurring in streptococci of group A, type 28 and in certain streptococci of other serological groups. J. Exp. Med. 96:83–97.
15. Lehner, T., S. J. Challacombe, and J. Caldwell. 1975. An immunological investigation into the prevention of caries in deciduous teeth of Rhesus monkeys. Arch. Oral Biol. 20:305–310.
16. McGhee, J. R., S. M. Michalek, J. Webb, J. M. Navia, A. F. R. Rahman, and D. W. Legler. 1975. Effective immunity to dental caries: protection of gnotobiotic rats by local immunization with *Streptococcus mutans*. J. Immunol. 114:300–305.
17. Miller, C. E., K. H. Wong, J. C. Feeley, and M. E. Forbines. 1972. Immunological conversion of *Vibrio cholerae* in gnotobiotic mice. Infect. Immun. 6:739–742.
18. Rantz, L. A., and E. Randall. 1955. Use of autoclaved extracts of hemolytic streptococci for serological grouping. Stanford Med. Bull. 13:290–291.
19. Robinet, H. G. 1962. Relationship of host antibody to fluctuations of *Escherichia coli* serotypes in the human intestine. J. Bacteriol. 84:896–901.
20. Sack, R. B., and C. E. Miller. 1969. Progressive changes of vibrio serotypes in germfree mice infected with *Vibrio cholerae*. J. Bacteriol. 99:688–695.
21. Scherp, H. W. 1971. Dental caries: prospects for prevention. Science 173:1199–1205.
22. Sirisinha, S. 1970. Reactions of human salivary immunoglobulins with indigenous bacteria. Arch. Oral Biol. 15:551–554.
23. Tanzer, J. M., G. J. Hageage, and R. H. Larson. 1973. Variable experiences in immunization of rats against *Streptococcus mutans*-associated dental caries. Arch. Oral Biol. 18:1425–1439.
24. Taubman, M. A. 1974. Immunoglobulins of human dental plaque. Arch. Oral Biol. 19:439–446.
25. Taubman, M. A., and D. J. Smith. 1974. Effects of local immunization with *Streptococcus mutans* on induction of salivary immunoglobulin A antibody and experimental dental caries in rats. Infect. Immun. 9:1079–1091.
26. Wadsworth, C. 1962. A microplate technique employing a gel chamber compared with other micro and macroplate techniques for immune diffusion. Int. Arch. Allergy 21:131–137.
27. Willers, J. M., and G. H. Alderkamp. 1967. Loss of type antigen in a type III *Streptococcus* and identification of the determinant disaccharide of the remaining antigen. J. Gen. Microbiol. 49:41–51.
28. Williams, R. C., and R. J. Gibbons. 1972. Inhibition of bacterial adherence by secretory immunoglobulin A: a mechanism of antigen disposal. Science 177:697–699.
29. Wilson, A. T. 1945. Loss of group carbohydrate during mouse passages of a group A hemolytic streptococcus. J. Exp. Med. 81:593–596.