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Effective Immunity to Dental Caries: Gastric Intubation of *Streptococcus mutans* Whole Cells or Cell Walls Induces Protective Immunity in Gnotobiotic Rats

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Gnotobiotic rats were given *Streptococcus mutans* 6715 whole cells (WC), purified cell walls (CW), or cell wall lysate by gastric intubation (GI), and assessments were made of humoral immune responses in serum and saliva and of caries protection. Levels of secretory immunoglobulin A (IgA) and IgG antibodies to *S. mutans* WC in saliva samples from experimental rats were determined by an enzyme-linked immunosorbent assay. Serum antibody levels of the IgM, IgG, and IgA isotypes were also determined. Similar levels of salivary antibodies were induced in rats given *S. mutans* WC or CW by GI, whereas lower salivary antibody titers were observed in rats given cell wall lysate by the oral route. The level of serum antibodies in the various groups of rats also reflected the oral antigen used. The specificity of salivary IgA and serum IgG antibodies in the various groups of rats was determined by enzyme-linked immunosorbent assay with lipoteichoic acid, serotype *g* carbohydrate, dextran, CW, and WC as coating antigens. Salivary IgA and serum IgG antibodies in rats given *S. mutans* WC or CW by GI were primarily directed to lipoteichoic acid and serotype *g* carbohydrate. The presence of salivary IgA antibodies to *S. mutans* in rats given either *S. mutans* WC or CW by GI correlated with a significant reduction in the levels of plaque, numbers of viable *S. mutans* in plaque, and caries scores when compared with the control animals (infected only). These results demonstrate that particulate antigens of *S. mutans* induce salivary immune responses when given by GI to gnotobiotic rats and that the presence of these antibodies correlates with caries protection.

Streptococcus mutans is the principal etiological agent of dental caries, and numerous studies have shown that the induction of salivary antibodies to various antigenic surface components of this bacterium results in caries immunity (reviewed in 20). Local injection of killed *S. mutans* whole cells (WC) in complete Freund adjuvant into the salivary gland region of rats induces a local salivary antibody response and caries immunity (21, 32). In these and in similar studies, the presence of secretory IgA (sIgA) antibodies in saliva correlated with caries reductions. Although promising, these procedures involving local injection of antigen in adjuvant are not suitable for human use, largely because of the adverse side effect of local inflammation which results from the use of strong adjuvants such as complete Freund adjuvant. Nevertheless, this local immunization model has been useful for evaluating *S. mutans* antigens that are important in caries immunity.

The most extensive studies to date with purified antigens of *S. mutans* for caries immunity are those of Taubman, Smith, and Ebersole (29–31, 33) with glucosyltransferase (GTF) and those of Russell, Lehner, and colleagues (16, 17, 28) with protein antigens. In these studies, the injection of purified GTF in adjuvant into the salivary gland region of rats or hamsters induced salivary IgA antibodies which could neutralize and bind GTF. The immunized experimental animals showed significant reductions in the incidence of caries (33). Studies by Lehner and his associates (16, 17) have largely involved a primate model and immunization of purified protein antigens by peripheral routes. Purified protein antigens of *S. mutans* induced significant protection in rhesus monkeys when administered by peripheral routes.

Numerous recent studies have indicated that the stimulation of gut-associated lymphoretic-

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ular tissue such as Peyer's patches with antigen results in sensitized lymphoid cells capable of populating distant mucosal tissues, including exocrine glands, e.g., salivary, lacrimal, and mammary tissues (5, 26, 37). Other studies have indicated that similar stimulation of bronchial-associated lymphoreticular tissue also provides precursor IgA B cells at mucosal sites (2, 18, 19, 27). Thus, a common immune defense system exists at our mucosal surfaces and is mobilized after natural antigen encounters, e.g., ingestion or inhalation of environmental antigens (reviewed in 20). This method of inducing secretory immune responses offers the most practical approach for eliciting protective salivary IgA antibodies to *S. mutans*. Our past studies (reviewed in 20) have shown that feeding *S. mutans* WC to gnotobiotic rats selectively induces sIgA antibodies in saliva as well as in colostrum and milk. Smith and co-workers (30, 31) have shown that oral administration of GTF induces sIgA antibodies to this antigen in saliva. The presence of salivary antibodies to *S. mutans* cell surface components correlates with a reduced incidence of caries.

Our long-range goals in this area of research have been directed along two broad lines. The first involves defining the surface antigens of *S. mutans* which exhibit lymphoproliferative activity and are suitable for inducing caries immunity after oral administration. The second related area involves evaluating adjuvants for enhancing sIgA responses to *S. mutans* antigens in external secretions such as saliva.

In the present study, we have determined the optimal conditions for gastric intubation (GI) of *S. mutans* antigens for inducing salivary IgA immune responses. We have compared the potential of particulate antigen forms, namely *S. mutans* WC and purified cell walls (CW), and of a soluble antigen form, namely cell wall lysate (CWL), for the induction of responses protective against *S. mutans*-induced dental caries.

MATERIALS AND METHODS

Gnotobiotic rats. Fischer rats (germfree CD F(344)GN; original breeders obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were used in all experiments. All rats were maintained in Trexler plastic isolators as previously described (24). Rat offspring (age, 19 days) were transferred to separate experimental isolators, treated (see below), and provided with sterile caries-promoting diet 305 and drinking water ad libitum.

Microorganism and immunogens. *S. mutans* 6715 (24) was employed in this study. Stock cultures were maintained at 4°C in brain heart infusion agar (Difco Laboratories, Detroit, Mich.) slabs containing excess calcium carbonate. For animal infection, cultures of *S. mutans* 6715 were grown in brain heart infusion broth (Difco) at 37°C in an atmosphere of 5% carbon dioxide and 95% nitrogen. An 18 h, broth culture of *S. mutans* 6715 was introduced into the isolator just before challenge (see below).

S. mutans WC immunogen was prepared as previously described (21). Breifly, *S. mutans* 6715 was grown in dialyzed tryptose medium (21). After 18 to 24 h of incubation at 37°C, the cells were harvested by centrifugation (4,000 × *g*, 30 min), washed five times with 0.1 M phosphate-buffered saline (pH 7.0), and suspended in 0.5% Formalin-saline. The killed cells were suspended in 0.1% Formalin-saline and stored at 4°C until used (see below).

Purified CW of *S. mutans* 6715 were obtained after mechanical disruption of WC with a Braun cell homogenizer, differential centrifugation, and proteolytic enzyme treatment of the cell pellet as previously described (13). Subsequent overnight incubation (37°C) of a portion of the purified CW preparation with the enzyme mutanolysin (38) yielded the CWL immunogen used in this study (12).

Experimental design. Before the immunization of rats, each immunogen was suspended to the appropriate dose (see below) in sterile intubation medium consisting of 8 parts Hanks balanced salt solution (GIBCO, Grand Island, N.Y.) and 2 parts sodium bicarbonate (7.5% solution, GIBCO) (15) and was transferred into each experimental isolator. Groups of rats (15 to 20 animals per group) were given the appropriate immunogen or (in the case of controls) intubation medium alone by GI with the aid of an intubation needle (Popper and Sons, Inc., New Hyde Park, N.J.) at 19, 20, 28, 35, and 42 days of age (Fig. 1). At 25 days of age, all rats (except uninfected controls) were challenged with 100 μl of an 18-h culture of *S. mutans* 6715 (4.5×10^6 to 5.0×10^6 CFU) with the aid of a micropipetter. Cages were changed twice daily after each oral immunization step to help ensure that antigen in feces would not be reintroduced into the oral cavity by coprophagy.

At the end of each experiment, rats were removed from the isolator, individual saliva and serum samples were collected (21), and mandibles were aseptically removed to quantitate plaque, levels of *S. mutans* in plaque, and caries scores (24). Each rat was weighed and injected with pentobarbital sodium (22, 24), and saliva was collected with the aid of a capillary pipette

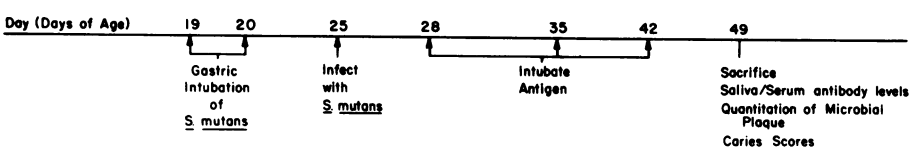

FIG. 1. Experimental design for studies of GI of either particulate or soluble *S. mutans* antigen for inducing salivary immune responses and caries immunity in gnotobiotic rats.

TABLE 1. Levels of anti-*S. mutans* 6715 in serum and saliva of gnotobiotic rats given *S. mutans* 6715 WC vaccine by GI

Treatment of rats	Antibody level[a]				
	Saliva		Serum		
	IgA	IgG	IgM	IgG	IgA
Infection only	<5.0	<5.0	<5.0	<5.0	<5.0
GI					
4×10^8 CFU/ml	7.7 ± 0.6	<5.0	<5.0	6.1 ± 1.1	<5.0
4×10^9 CFU/ml	52.8 ± 3.8	11.4 ± 0.5	<5.0	9.2 ± 0.7	16.1 ± 0.4
4×10^{10} CFU/ml	9.6 ± 0.8	<5.0	<5.0	<5.0	7.1 ± 0.3

[a] Determined by ELISA with optimal concentrations of monospecific anti-rat α, γ, or μ heavy-chain-specific sera. Values are expressed as the mean ± standard error of the mean of ELISA units where one ELISA unit equals the mean reciprocal of the dilution of triplicate tests per sample per group (15 to 20 rats per group; see legend to Fig. 2) giving an optical density (OD) reading at 405 nm of 0.1 after 1.5 h of incubation with substrate.

after pilocarpine stimulation (22). A 1-ml portion of whole saliva was collected from each rat over a 30-min interval. The samples were centrifuged (2,800 × *g*, 15 min) and stored at −20°C until assayed for antibody activity (see below). After saliva collection, rat blood was obtained by cardiac puncture; blood was allowed to clot at room temperature and incubated overnight at 4°C. Serum was collected after centrifugation (4,400 × *g*, 15 min) and stored at −20°C until assayed. Mandibles from individual rats were aseptically removed and defleshed. The left mandible from each rat was stained with safranin and scored for plaque as previously described (24). The right mandible was transferred to a tube containing sterile phosphate buffer (0.067 M, pH 7.0; 3 ml). The plaque was disrupted from molar surfaces by sonication (Sonifier cell disruptor; Branson Instruments Co., Plain View, N.Y.), and the numbers of *S. mutans* (and absence of contaminating microorganisms) in plaque were determined after cultivation of samples on blood and Mitis-Salivarius agar as previously described (24). The mandibles were then stained with murexide (0.4% in 70% ethanol) and hemisectioned, and buccal, sulcal, and proximal molar caries were scored by the Keyes procedure as modified by our laboratory (24).

Antibody assay. The level, isotype, and specificity of antibody in saliva and serum samples from experimental animals were determined by an enzyme-linked immunosorbent assay (ELISA) (10, 36). Briefly, 200 μl of antigen suspension or solution in 0.1 M carbonate buffer (pH 9.6) was added to wells of flat-bottom polystyrene microtiter plates (Linbro EIA; Flow Laboratories, Inc., McLean, Va.). After overnight incubation at 37°C, unbound antigen was removed by repeat-

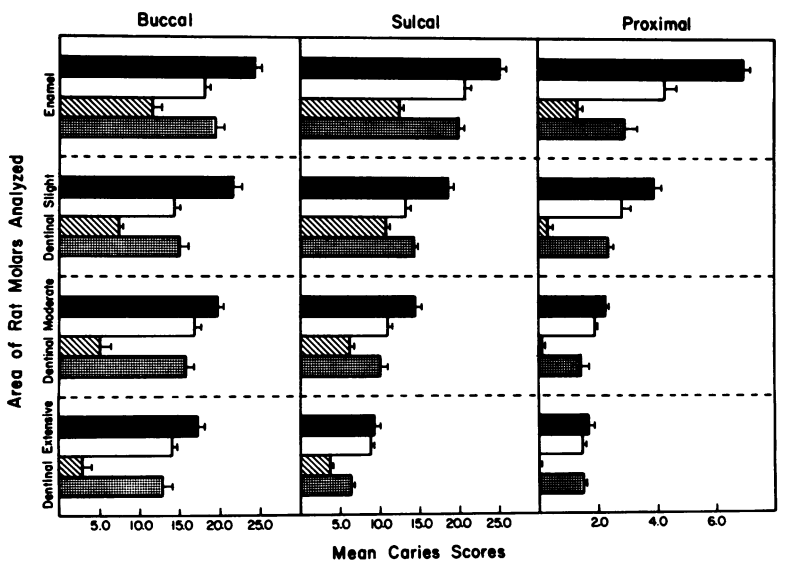

FIG. 2. Caries scores on buccal, sulcal, and proximal molar surfaces of groups of gnotobiotic rats (age, 49 days) infected with *S. mutans* 6715 only (■, 20 rats) or given 10^8 (□, 15 rats), 10^9 (▨, 18 rats), or 10^{10} (▦, 20 rats) equivalent *S. mutans* 6715 CFU by GI (Fig. 1) and infected at age of 25 days with viable *S. mutans* 6715. Values shown are means ± standard errors of the mean.

TABLE 2. Increase in levels of anti-*S. mutans* 6715 antibodies in serum and saliva of gnotobiotic rats given *S. mutans* 6715 WC vaccine by GI

Age (days)	Antibody level[a]									
	Saliva				Serum					
	Infected		Control		Infected			Control		
	IgA	IgG	IgA	IgG	IgM	IgG	IgA	IgM	IgG	IgA
25	20.0 ± 0.8	8.3 ± 0.4	19.4 ± 0.2	8.5 ± 1.1	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0
28	19.5 ± 0.5	8.0 ± 0.2	20.1 ± 0.3	8.7 ± 0.9	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0
35	28.1 ± 1.4	11.2 ± 0.3	22.4 ± 0.2	10.0 ± 1.2	<5.0	<5.0	10.0 ± 0.4	<5.0	<5.0	<5.0
42	34.6 ± 0.7	10.8 ± 0.2	25.2 ± 0.9	11.2 ± 0.5	<5.0	<5.0	12.6 ± 0.3	<5.0	<5.0	<5.0
49	42.5 ± 2.4	12.1 ± 0.3	29.7 ± 1.4	10.4 ± 0.3	<5.0	10.4 ± 0.4	15.8 ± 0.3	<5.0	<5.0	9.7 ± 0.2

[a] Determined by ELISA with optimal concentrations of monospecific anti-rat α, γ, or μ heavy-chain-specific sera. Values are expressed as the mean ± standard error of the mean of ELISA units where one ELISA unit equals the mean reciprocal of the dilution of triplicate tests per sample per group (seven rats per group at each time interval) giving an OD reading at 405 nm of <0.1 after 1.5 h of incubation with substrate.

ed washing with saline containing 0.05% Tween 20 (Tween-saline). Tween-saline was used in all washing steps and as diluent buffer. After the final wash, 200 μl of 1% bovine serum albumin in Tween-saline was added to individual wells and incubated at 25°C for 90 min. Plates were again extensively washed, and appropriate dilutions of either saliva or serum were added to wells (100 μl per well). After incubation (25°C, 3 h), plates were washed three times, and alkaline phosphatase-labeled rabbit anti-rat μ, γ, or α heavy-chain-specific antibody was added to the appropriate wells (21, 36). Plates were incubated overnight at 4°C and washed, and *p*-nitrophenylphosphate substrate (Sigma 104 phosphatase substrate; Sigma Chemical Co., St. Louis, Mo.) dissolved in diethanolamine buffer (pH 9.8, 1 mg/ml) was added (100 μg per well). The amount of color which developed after incubation at 25°C for 1.5 h was measured at 405 nm in the microtiter plate with a Titertek Multiskan photometer (Flow Laboratories).

Purified *S. mutans* CW components. *S. mutans* WC and CW (described above) and the following CW antigens were employed for assessment of antibody levels by ELISA. Serotype *g* carbohydrate (CHO *g*) was prepared from lyophilized *S. mutans* 6715 WC by a modified Rantz-Randall method. Briefly, 10 g (dry weight) of *S. mutans* 6715 WC was suspended in 200 ml of saline and autoclaved (121°C) for 30 min. Cells were removed by centrifugation, the supernatant removed and saved, and the cell sediment was subjected to this same treatment twice more. Supernatants were pooled, dialyzed exhaustively against distilled water, and concentrated by rotary perevaporation. The concentrated extract was applied to a DEAE–Sephadex A-25 column to remove nucleic acids and teichoic acid present. Unadsorbed fractions containing anthrone-reactive substances were pooled, concentrated, and fractionated on Bio-Gel P-100. The void volume fraction which reacted with an anti-serotype *g* antiserum was collected, lyophilized, and designated RR *g*. This preparation consisted of over 98% carbohydrate (manuscript submitted for publication). Furthermore, this preparation formed a single precipitin band with polyvalent rabbit anti-*S. mutans* 6715 sera which also exhibited reactivity to dextran, lipoteichoic acid (LTA), GTF, and cell surface protein (manuscript submitted for publication).

LTA was prepared by a modification of the phenol-water extraction method of Moskowitz (25). Briefly, *Streptococcus pyogenes* MJM 14 WC (400 mg) were suspended in distilled water (20 ml), and an equal volume of 90% phenol was then added. After incubation at 25°C for 1 h with occasional shaking, the water phase was removed, and the extraction procedure was repeated by adding 20 ml of distilled water to the phenol phase. The combined water phases were dialyzed exhaustively against distilled water, concentrated, and fractionated on a Sepharose 4B column. Fractions reactive with anti-polyglycerol phosphate were combined, dialyzed against distilled water, and lyophilized. This preparation formed a single precipitin line with polyvalent anti-*S. mutans* 6715 sera; however, no precipitin line was observed after adsorption of the sera with *S. pyogenes* WC (manuscript submitted for publication). Dextran T-2000 (Pharmacia Fine Chemicals, Piscataway, N.J.) was further purified by fractionation on Sepharose 2B, and the peak

TABLE 3. Levels of anti-*S. mutans* 6715 antibodies in serum and saliva of gnotobiotic rats given *S. mutans* 6715 WC, CW, or CWL by GI

Treatment of rats	Antibody level[a]				
	Saliva		Serum		
	IgA	IgG	IgM	IgG	IgA
Infection only	<5.0	<5.0	<5.0	<5.0	<5.0
GI					
WC	44.1 ± 2.2	12.6 ± 0.6	<5.0	6.4 ± 0.5	21.1 ± 0.4
CW	36.4 ± 3.4	9.6 ± 0.4	<5.0	8.6 ± 0.3	19.7 ± 0.5
CWL	8.5 ± 0.7	<5.0	<5.0	15.5 ± 0.2	<5.0

[a] Determined by ELISA with optimal concentrations of monospecific anti-rat α, γ, or μ heavy-chain-specific sera. Values are expressed as the mean ± standard error of the mean of ELISA units where one ELISA unit equals the mean reciprocal of the dilution of triplicate tests per sample per group (15 to 20 rats per group; see legend to Fig. 3) giving an OD reading at 405 nm of 0.1 after 1.5 h of incubation with substrate.

corresponding to a molecular weight of 2×10^6 was used in these studies.

Statistics. The caries scores from each group of rats were statistically reduced by computing means and standard errors. Differences among means were evaluated by an analysis of variance and by multiple mean comparisons by the Duncan test (9). The results of the plaque scores and the number of *S. mutans* CFU per rat mandible were expressed as the mean ± standard error. The significance of difference between means was determined by the Student *t* test.

RESULTS

Optimal conditions for the induction of salivary immune responses after oral administration of antigen. Our past oral immunization studies involved feeding *S. mutans* WC antigen to gnotobiotic rats (reviewed in 20). Therefore, an important aspect of the present study was to establish the optimal antigen doses for inducing caries immunity after the GI of antigen, a method which allows us to bypass the oral cavity. To establish the optimal dose of *S. mutans* 6715 WC required to induce a salivary immune response, groups of rats were given 10^8, 10^9, or 10^{10} equivalent *S. mutans* CFU by GI (Fig. 1), and the level and isotype distribution of the salivary and serum anti-*S. mutans* 6715 antibody response were assessed by ELISA (Table 1). Optimal salivary antibody responses were observed in rats given antigen doses of 10^9 equivalent CFU; sIgA represented the principal antibody isotype induced, although some IgG antibody could be detected. Low antibody levels of both IgG and IgA isotypes were observed in serum samples from this group of rats (Table 1). The antigen dose was critical since rats given either 10^8 or 10^{10} equivalent CFU by GI elicited either low or no salivary or serum immune response. The IgA antibodies induced in saliva were secretory in nature since antibody activity could be blocked with excess anti-secretory component; furthermore, most salivary IgA antibody activity eluted in an 11S fraction from a Sephadex G-200 column (data not shown).

The presence of sIgA antibodies in the saliva of rats receiving 10^9 equivalent *S. mutans* CFU correlated with reduced levels of *S. mutans*-induced dental caries (Fig. 2). On the other hand, rats which received the other antigen doses (either 10^8 or 10^{10} equivalent CFU) and which exhibited low salivary antibody levels

TABLE 4. Specificity of IgA anti-*S. mutans* 6715 antibodies in saliva of gnotobiotic rats given *S. mutans* 6715 WC, CW, or CWL by GI

Treatment of rats	IgA antibody level[a]				
	WC	CW	LTA	CHO (RR *g*)	Dextran T-2000
Infection only	<5	<5	9 ± 2	<5	<5
GI					
WC	74 ± 6	47 ± 4	53 ± 4	26 ± 4	<5
CW	66 ± 3	54 ± 8	14 ± 3	30 ± 5	23 ± 4
CWL	15 ± 5	16 ± 6	18 ± 4	<5	8 ± 2

[a] Determined by ELISA with an optimal concentration of monospecific anti-rat α-chain-specific serum. Values are expressed as the mean OD reading at 405 nm ($\times 10^{-3}$) ± standard error of the mean of quadruplicate tests per sample (1/15 dilution of original) per group (15 to 20 rats per group; see legend to Fig. 3) after 1.5 h of incubation with substrate.

TABLE 5. Specificity of IgG anti-*S. mutans* 6715 antibodies in serum of gnotobiotic rats given *S. mutans* 6715 WC, CW, or CWL by GI

Treatment of rats	IgG antibody level[a]				
	WC	CW	LTA	CHO (RR *g*)	Dextran T-2000
Infection only	<5	<5	18	<5	6
GI					
WC	25 ± 5	37 ± 4	50 ± 4	16 ± 2	29 ± 3
CW	32 ± 3	42 ± 6	18 ± 3	18 ± 2	31 ± 5
CWL	43 ± 5	49 ± 5	23 ± 3	14 ± 2	31 ± 4

[a] Determined by ELISA with an optimal concentration of monospecific anti-rat γ-chain-specific serum. Values are expressed as the mean OD reading at 405 nm ($\times 10^{-3}$) ± standard error of the mean of quadruplicate tests per sample (1/25 dilution of original) per group (15 to 20 rats per group; see legend to Fig. 3) after 1.5 h of incubation with substrate.

showed only slight reductions in caries lesions on all molar surfaces (Fig. 2). In rats which had received 10^9 equivalent *S. mutans* CFU, both the level and degree of caries seen on all three molar surfaces were significantly lower ($P \leq 0.01$) than those obtained in the controls (infected only) or in rats given either 10^8 or 10^{10} equivalent CFU by GI. These results clearly demonstrate that the GI of 10^9 *S. mutans* 6715 equivalent CFU to gnotobiotic rats induced salivary antibody responses (largely sIgA responses) which correlated with protection against *S. mutans*-induced disease.

In the next series of experiments, the kinetics of the immune response after the GI of 10^9 equivalent CFU was determined (Table 2). Two groups of animals were orally immunized at the indicated times (Fig. 1); one group was challenged with *S. mutans* 6715 on day 25, whereas the second group was not challenged with living cells. Rats were removed from the experiment at the indicated times (Table 2), and the levels of both salivary and serum antibodies were assessed by ELISA. Antibodies to *S. mutans* could be detected in saliva as early as 6 days after the first immunization, and antibody levels, most notably of the sIgA isotype, steadily increased with time throughout the experiment. These results indicate that antibodies were present in saliva at the time of challenge with viable *S. mutans* (day 25). Levels of sIgA became significantly higher ($P \leq 0.05$) in immunized and infected rats than in the immunized and noninfected animals (Table 2). The other important point deserving emphasis was that salivary IgG antibodies were detectable by day 25; however, the level did not markedly increase during the experiment. Low levels of serum antibodies, especially of the IgA isotype, were noted after the third administration of antigen (Table 2).

Importance of antigen form for inducing immune responses and caries immunity after oral administration of *S. mutans* antigens. The next experiments were conducted to compare immune responses in serum and saliva of rats after the GI of *S. mutans* 6715 purified CW or CWL with immune responses after administering *S. mutans* 6715 WC. In this study, the relative immunogenicity of particulate and soluble CW components for the induction of mucosal (saliva) versus systemic (serum) responses was assessed. Groups of 15 to 20 rats were given *S. mutans* 6715 WC (10^9 equivalent CFU), CW (0.5 mg), or CWL (0.5 mg) by GI according to the experimental design outlined in Fig. 1. The doses of CW and CWL were chosen to approximate an equivalent dose of WC (dry weight). When levels of anti-*S. mutans* 6715 antibodies were assessed in saliva and serum samples, rats given either WC or CW exhibited similar levels of sIgA or IgG antibodies in saliva (Table 3). CWL, on the other hand, induced poor salivary antibody responses; sIgA antibody levels were approximately fourfold less than those seen in rats orally immunized with the particulate antigens WC or CW (Table 3). IgA also represented the major antibody isotype induced in the serum of rats receiving either WC or CW; however, some IgG antibodies were also observed. Rats which received CWL by GI mainly exhibited serum IgG antibodies. These results indicate that particulate *S. mutans* antigens (WC or CW) induced sIgA responses (in saliva) and serum IgA antibodies, whereas the soluble antigen (CWL) induced significantly ($P \leq 0.01$) lower salivary and serum IgA responses and twofold higher serum IgG responses.

When the spectrum of sIgA antibody specificities in saliva was determined, a similar pattern was seen in rats which had been orally immunized with the particulate antigen forms (Table 4). Comparable levels of sIgA antibodies from both groups of rats reacted with WC, CW, and CHO *g*; however, rats given WC by GI had significantly higher ($P \leq 0.05$) sIgA antibody levels to LTA. Rats which received either CW or CWL exhibited lower antibody levels to this antigen, probably because significant amounts

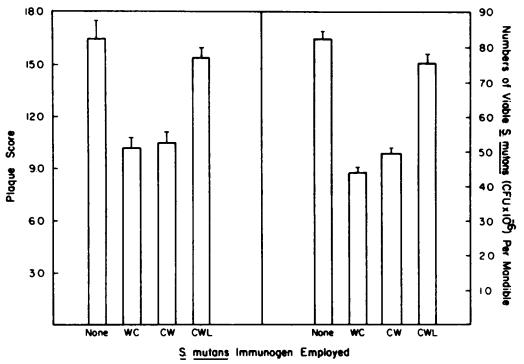

FIG. 3. Plaque scores and number of viable *S. mutans* organisms in plaque of gnotobiotic rats (age, 49 days) infected with *S. mutans* 6715 only (None; 15 rats) or given *S. mutans* 6715 WC (18 rats), CW (17 rats), or CWL (20 rats) antigen by GI and infected at age of 25 days with viable *S. mutans* 6715. Values shown are the means ± standard errors of the mean.

of LTA were lost during the purification of CW. These results also suggest that LTA is an important immunogen in *S. mutans* 6715 WC and induces significant sIgA antibodies in saliva when administered by the oral route. Rats given CW (and to a lesser extent CWL) by GI exhibited sIgA antibodies to dextran T-2000 (Table 4); however, rats given WC did not respond to this antigen.

Serum IgG antibodies exhibited a similar pattern of specificity to the *S. mutans* antigens (Table 5). As described above, CWL induced high levels of IgG antibodies which reacted with WC and lower levels of antibodies to CW. Moderate levels of antibodies to LTA, CHO *g*, and dextran were also seen. Rats orally immunized with either WC or CW showed similar levels of serum IgG antibodies to WC, CW, CHO *g*, and dextran T-2000.

When plaque and viable numbers of *S. mutans* 6715 in plaque were assessed in rats orally immunized with the various antigens and compared with controls (infected with *S. mutans* 6715 only), significant ($P \leqq 0.01$) reductions in levels were seen in rats receiving particulate antigen (WC or CW) (Fig. 3). The reductions in plaque and numbers of detectable *S. mutans* in plaque correlated with elevated sIgA antibodies induced in these rats. Conversely, the presence of low levels of salivary antibodies in rats given soluble antigen (CWL) orally did not effectively diminish plaque formation by *S. mutans*.

When levels of caries were assessed in these groups, rats given either *S. mutans* WC or CW by GI exhibited significantly lower ($P \leqq 0.01$) caries activity on buccal, sulcal, and proximal molar surfaces than did control rats (infected only) or rats given *S. mutans* CWL antigen (Fig. 4). Although the level of caries in rats given *S. mutans* CWL was slightly lower than that obtained in control animals (infected only), the difference in the extent of decay (dentinal moderate and extensive) between these two groups was not significantly different ($P \leqq 0.01$). Thus, caries immunity resulted in rats with significant sIgA antibodies in saliva. These antibodies effectively reduced plaque and levels of *S. mutans* in plaque and resulted in effective caries immunity (Fig. 3 and 4).

DISCUSSION

A major finding in the present study is the observation that direct administration of *S. mutans* antigens into the stomach of gnotobiotic rats by gavage, a procedure purposely selected to bypass the oral cavity, resulted in the induction of salivary antibodies. A significant portion of the salivary immune response was represented by sIgA. The response pattern was dose dependent since 10^9 equivalent *S. mutans* 6715 CFU induced significant responses in saliva, but doses one log lower (10^8 equivalent CFU) or higher (10^{10} equivalent CFU) did not. This lack of significant response in rats given either 10^8 or 10^{10} equivalent CFU orally was probably not due to an inability to detect salivary antibodies since the ELISA employed with *S. mutans* antigens is quite sensitive (1, 3, 4, 11, 30). Thus, the dose of antigen required to induce the response appears to be critical and should be carefully considered when studies of this nature are undertaken. Our past studies (23) have indicated that when *S. mutans* WC are fed in diet to gnotobiotic rats, higher levels of antigen also result in an unresponsive state. It is important for us to establish whether the GI of higher doses of *S. mutans* immunogen results in systemic unresponsiveness (oral tolerance; 34). In elegant experiments, Challacombe and Tomasi (6) have found that the oral administration of *S. mutans* to mice induces both systemic unresponsiveness (oral tolerance) and IgA responses in saliva. We are currently measuring serum responses to antigen administered intravenously to rats which had received different doses of *S. mutans* orally to determine whether oral tolerance is induced by the immunization regimen.

A second important aspect of the present study is the observation that significant antibody responses occurred in the saliva of young rats five or six days after the GI of *S. mutans* (Table 2). These results clearly indicate that local antibodies were induced and were present in saliva at the time of *S. mutans* challenge. Nevertheless, colonization of the organism occurred in the oral cavities of these rats and contributed to plaque formation and caries, albeit at significant-

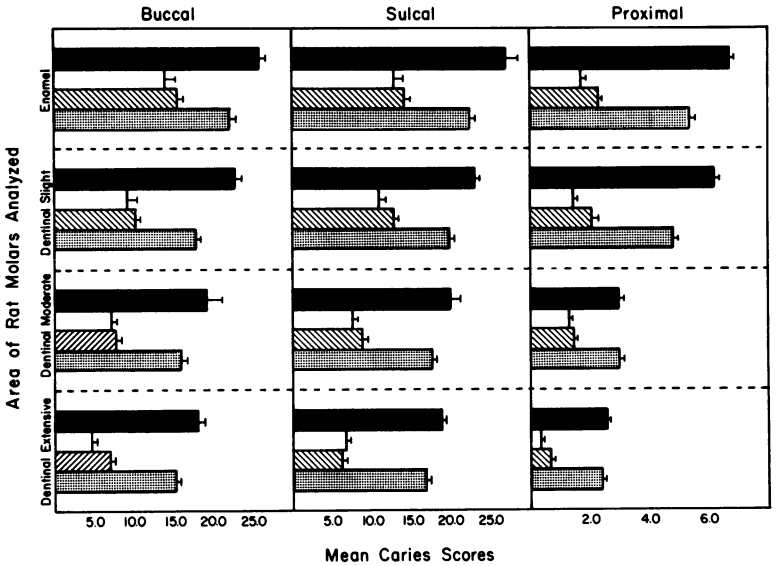

FIG. 4. Caries scores on buccal, sulcal, and proximal molar surfaces of groups of gnotobiotic rats (age, 49 days) infected with *S. mutans* 6715 only (■, 15 rats) or given *S. mutans* 6715 WC (□, 18 rats), CW (▨, 17 rats), or CWL (▦, 20 rats) by GI and infected at age of 25 days with viable *S. mutans* 6715. Values are the means ± standard errors of the mean.

ly lower levels than those seen in infected and nonimmunized controls. The presence of *S. mutans* in the oral cavities of rats receiving antigen by the gastric route may actually contribute to the local immune process. Husband and Gowans (14) have shown that precursor IgA B cells home to mucosal tissue in the absence of locally present antigen; however, greater numbers of IgA-producing cells were seen in tissue which had been supplied with antigen. Thus, precursor cells for IgA responses to *S. mutans* may be induced in gut-associated lymphoreticular tissue and home (via mesenteric lymph nodes, thoracic duct lymph, and the bloodstream) to the oral cavity (salivary glands). In this site, cells may await further differentiation signals supplied by antigen locally present in this tissue. We have addressed this point in the current paper, and our evidence suggests that continued oral administration of antigen induces progressively higher sIgA immune responses in saliva. However, sIgA responses were always higher in rats which had been orally colonized with *S. mutans* (Table 2). Thus, as others have suggested (14), the presence of antigen locally may ensure the survival of IgA B cells and the longevity of the local immune response. Additional studies will be required to establish that greater numbers of plasma cells synthesizing antibodies to *S. mutans* antigens are present in the salivary glands of rats infected with *S. mutans*, and studies of this nature are currently in progress.

Rats given either particulate or soluble forms of *S. mutans* antigens by GI also produced low but detectable levels of serum antibodies. Of interest was the finding that particulate forms induced largely IgA antibodies, whereas a soluble form (CWL) induced principally serum IgG antibodies. It is tempting to speculate that soluble antigen reaches peripheral tissue such as spleen and induces IgG responses there. If this occurs, one might also expect serum IgM responses; however, this isotype response was not detected by ELISA. Perhaps more important was the finding that particulate antigen forms, i.e., WC and CW, induced largely sIgA responses in saliva as well as serum responses of the same isotype. It is not known whether the serum form of IgA is polymeric; however, studies along these lines are in progress. Nevertheless, our results clearly suggest that particulate forms of *S. mutans* antigens are effective for inducing sIgA responses in saliva. It should be pointed out that Cox and Taubman (7, 8) have demonstrated that the injection of a soluble antigen at sites distant from the salivary gland region is more effective than a particulate antigen form in priming the host for a subsequent salivary IgA response after antigen injection in the region of the salivary glands. We are currently testing this possibility with soluble antigens derived from *S. mutans* CW.

As for the specificities of antibodies induced, sIgA in the saliva of rats orally immunized with particulate antigens appeared to be directed to major determinants of the CW. WC antigen

induced sIgA antibodies reactive with purified CW and two major purified CW components, CHO *g* and LTA. On the other hand, particulate CW induced sIgA antibodies mainly to CHO *g* and dextran. Since the CW purification procedure employed depletes the preparation of major portions of LTA, this immunogen form induced lower titers of sIgA with specificity to LTA than were found when WC was used for immunization. Nevertheless, some antibodies to LTA were seen, suggesting indirectly that LTA, when present (even in small amounts) in a particulate form, is immunogenic when given by the oral route. It remains to be established whether CW results in significant antibody responses to the peptidoglycan backbone. Nevertheless, it is clear that purified CW, which contains peptidoglycan, serotype CHO, and dextran, induces significant sIgA antibodies in the saliva of rats orally immunized with this antigen and that the induced antibodies are effective in caries immunity. Therefore, serious consideration must be given to purified *S. mutans* CW as a candidate for use in the production of an effective caries vaccine (20).

Our study suggests that the serotype CHO antigen may be an important determinant for inducing protective salivary antibodies since both WC and CW induced significant sIgA responses to this determinant. Interestingly, the solubilized CW, which contains significant amounts of this CHO antigen, did not induce elevated levels of salivary antibodies to this antigen, and rats from this group were not significantly protected from *S. mutans*-induced disease. Our past studies (12, 35) have clearly suggested that CHO antigen is an effective murine B cell mitogen and polyclonal B cell activator and that haptenated forms induce thymic-independent immune responses. Since it is now established that IgA responses to most antigens are thymic dependent, it is possible that serotype CHO in a particulate form (in the CW) acts as a thymic-dependent antigen for IgA responses. Studies directed to determine the thymic-dependent or thymic-independent nature of this antigen in the CW are currently under way.

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