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Effective Immunity to Dental Caries: Enhancement of Salivary Anti-*Streptococcus mutans* Antibody Responses with Oral Adjuvants

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In the present study, we compared the ability of the soluble adjuvants concanavalin A (ConA), muramyl dipeptide (MDP), and peptidoglycan (PG) to enhance immune responses to orally administered particulate antigens of *Streptococcus mutans* 6715 in gnotobiotic rats. The isotype and levels of antibody in saliva and in serum from experimental rats were determined by an enzyme-linked immunosorbent assay using *S. mutans* whole cells (WC) as the coating antigen. The specificities of salivary and serum immunoglobulin A (IgA) antibodies to particulate *S. mutans* antigens, lipoteichoic acid, *S. mutans* serotype *g* carbohydrate, and dextran were also determined. When 50 μg of ConA was used as the oral adjuvant with *S. mutans* 6715 WC immunogen, a slight enhancement of immune responses was obtained. A higher dose of ConA suppressed humoral responses to the immunogen. Enhanced immune responses, especially of the IgA isotype, in both serum and saliva were induced in gnotobiotic rats given MDP and either *S. mutans* 6715 WC or purified cell walls (CW) by gastric intubation. Elevated IgA antibody levels to CW, lipoteichoic acid, and carbohydrate were observed in rats given *S. mutans* WC and MDP by gastric intubation, whereas oral immunization with *S. mutans* CW and MDP resulted in higher antibody levels to CW and carbohydrate and lower levels to lipoteichoic acid when compared with the antibody levels in rats given antigen alone. Rats orally immunized with either *S. mutans* WC or CW and MDP and challenged with virulent *S. mutans* 6715 exhibited significantly ($P \leq 0.05$) lower plaque scores, numbers of viable *S. mutans* in plaque, and caries scores than did rats immunized with antigen alone or in infected-only controls. In another series of experiments, a PG fraction derived from *S. mutans* 6715 CW was assessed for adjuvant properties. The oral administration of PG and either *S. mutans* WC or CW induced good salivary and serum IgA antibody responses. The specificity of the antibodies was similar to that obtained in rats given antigen and MDP. Rats receiving either *S. mutans* WC or CW and PG and challenged with virulent *S. mutans* 6715 had lower plaque scores, fewer numbers of viable *S. mutans* in plaque, and lower caries activity than did infected rats receiving *S. mutans* WC or CW immunogen alone. These results provide evidence that soluble adjuvants derived from the gram-positive bacterial CW, e.g., MDP and PG, are effective oral adjuvants and augment IgA immune responses to particulate *S. mutans* antigens which are protective against the mucosally associated disease, dental caries.

The demonstration that *Streptococcus mutans* is the principal etiological agent in human dental caries (18, 19) has resulted in numerous investigations to ascertain the major virulence determinants of this bacterium and the most effective means for inducing salivary antibodies to *S. mutans* surface components, which protect the host from the infectious disease dental caries (reviewed in reference 21). Studies aimed at developing an effective caries vaccine generally have been directed along two broad lines. The first approach has involved the elucidation of the major cell surface antigens of *S. mutans* most suitable for inducing salivary antibodies which protect against infection (reviewed in reference 21). The most extensive studies to date with

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purified antigens of *S. mutans* for caries immunity are those of Taubman and co-workers (35–39) with glucosyltransferase (GTF) and those of Russell, Lehner, and their colleagues (15, 16, 34) with protein antigens. The injection of purified GTF in complete Freund adjuvant into the salivary gland region of rats or hamsters induced salivary IgA antibodies with GTF inhibiting and binding properties. These immunized animals had significantly lower caries scores than did the infected controls (37).

The second major approach has concerned the route of *S. mutans* antigen administration, since the use of local adjuvants, such as complete Freund adjuvant, results in significant inflammation at the injection site and, thus, is unsuitable for human use. In this regard, Lehner and co-workers (2, 15–17) have shown that the systemic immunization of primates with *S. mutans* antigens elicits the production of significant serum immunoglobulin M (IgM), IgA, and especially IgG antibodies which reach dental plaque via the crevicular exudate and result in caries immunity.

In recent years, studies have suggested that oral immunization with *S. mutans* antigen can result in the induction of salivary IgA antibodies and protection against *S. mutans*-induced dental caries in gnotobiotic rats (24, 25). These studies and those of others (reviewed in reference 21) indicate that the oral administration of antigen results primarily in the stimulation of lymphoid cells in gut-associated lymphoreticular tissue (GALT) (1, 33, 42). Sensitized cells, including precursor IgA B cells, leave GALT and follow a circuitous route, including mesenteric lymph nodes → thoracic duct lymph → peripheral blood before settling in mucosal tissues (33, 42), including salivary glands. The final differentiation into plasma cells synthesizing IgA antibodies with specificity for gut-encountered antigens provides the host with a mucosal immune defense system common to most, or perhaps all, secretory tissues.

Previous studies have demonstrated that the provision of *S. mutans* antigen in water or diet (24, 26) or weekly by gastric intubation (GI) (27) induces salivary IgA immune responses and caries protection. These results, as well as studies by others (35–37, 39), have shown that the induction of salivary IgA antibodies to *S. mutans* antigens results in significant protection against caries formation. However, complete protection against caries formation has not been observed. Therefore, our laboratory has begun to assess appropriate adjuvants, suitable for oral administration, to determine whether an enhancement of IgA responses could be attained. In this regard, previous studies have shown that the soluble molecular adjuvants concanavalin A (ConA), lipopolysaccharide (LPS), and muramyl dipeptide (MDP) enhance in vitro immune responses to thymic-dependent antigens in murine Peyer patch (PP) cell cultures (12, 13). Furthermore, the oral administration of ConA with either bacterial (14) or erythrocyte (11) antigen results in enhanced murine IgA responses. Studies by Taubman and co-workers (38) indicate that GI of GTF and MDP to rats results in elevated salivary IgA antibody responses when compared with animals given GTF only.

In the present study, we evaluated the soluble adjuvants ConA, MDP, and peptidoglycan (PG) for their ability to enhance immune responses to particulate *S. mutans* antigens given by the oral route. The oral administration of either MDP or PG and *S. mutans* antigen enhanced salivary IgA responses and resulted in greater caries protection than was observed in animals given antigen alone.

MATERIALS AND METHODS

Rats. Germfree Fischer rats [CD F(344)GN; original breeders obtained from Charles River Laboratories, Wilmington, Mass.] were used in all experiments. Rat offspring (age, 19 days) were transferred to experimental Trexler plastic isolators, treated (see below), and provided sterile caries-promoting diet 305 and drinking water ad libitum, as previously described (26).

Microorganism and immunogen. Stock cultures of *S. mutans* 6715 were maintained at 4°C in brain heart infusion agar (Difco Laboratories, Detroit, Mich.) stabs containing excess calcium carbonate. For the infection of germfree rats, cultures of *S. mutans* 6715 were grown in brain heart infusion broth (Difco) at 37°C in a 5% CO_2-in-nitrogen atmosphere. An 18-h broth culture of *S. mutans* 6715 was introduced into appropriate isolators immediately before challenge (see below and Fig. 1).

For the preparation of the immunogen, *S. mutans* 6715 was grown in dialyzed tryptose medium (22) for 18 to 24 h at 37°C. The cells were harvested (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 whole cells (WC) were suspended in 0.1% Formalin-saline and stored at 4°C until used (see below).

Purified cell walls (CW) of *S. mutans* 6715 were obtained after mechanical disruption of washed WC, using a Braun cell homogenizer; differential centrifugation; and proteolytic enzyme, RNase, and DNase treatment of the cell pellet, as previously described (11).

Adjuvants. ConA (Miles Laboratories, Inc., Elkhardt, Ind.), MDP (*N*-acetylmuramyl-L-alanyl-D-isoglutamine; adjuvant peptide; Sigma Chemical Co., St. Louis, Mo.), and a PG-containing fraction of *S. mutans* (see below) were dissolved in intubation medium consisting of eight parts Hanks balanced salt solution (GIBCO Laboratories, Grand Island, N.Y.) and two parts sodium bicarbonate (7.5% solution; GIBCO) (11), appropriately diluted, and mixed with *S. mutans* 6715 antigen (see below) before oral immunization. The PG fraction was prepared from purified *S. mutans*

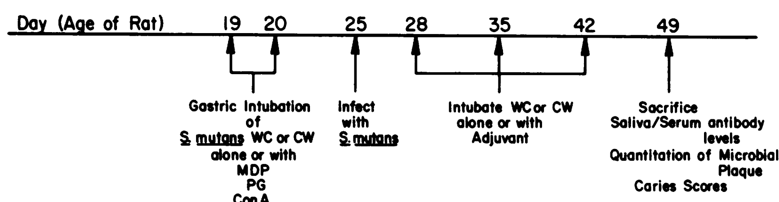

FIG. 1. Experimental design employed in studies of GI of particulate *S. mutans* antigens and molecular adjuvants for augmentation of salivary immune responses and enhanced caries protection in gnotobiotic rats.

6715 CW by treatment with mutanolysin enzyme (1 mg of enzyme per 50 mg of CW) at 37°C for 48 h, as previously described (10). Nonlysed CW were removed by centrifugation (15,000 × *g*, 30 min), and the supernatant was collected, dialyzed exhaustively against distilled water, and lyophilized. The PG was purified by carboxymethyl cellulose-Sephadex C-25 and Sephadex G-100 column chromatography. The second peak from Sephadex G-100, which corresponded to a lower-molecular-weight material, was collected, desalted by Sephadex G-25 column chromatography, and lyophilized. This material consisted mainly of glucosamine, muramic acid, and amino acids associated with PG, as well as rhamnose and glucose; however, no serotype carbohydrate, protein, or lipoteichoic acid (LTA) was detected (S. Hamada, M. Torii, N. Okahashi, S. Kowata, K. Yokogawa, S. M. Michalek, and J. R. McGhee, Microbiol. Immunol., in press).

Experimental design. Before immunization, *S. mutans* 6715 WC (4×10^9 equivalent CFU/ml) or CW (2.0 mg/ml) was suspended in sterile intubation medium with or without an adjuvant. Groups of rats (12 to 18 animals per group) were given the appropriate immunogen, immunogen and adjuvant, adjuvant alone, or intubation medium alone by GI (see below) with the aid of an intubation needle (Popper and Sons, Inc., New Hyde Park, N.J.) on 19 and 20 days of age (Fig. 1). Since the admixture of ConA with *S. mutans* 6715 WC resulted in agglutination, it became necessary to intubate ConA before oral antigen administration. In these experiments, ConA was administered 4 h before *S. mutans* antigen. At the age of 25 days, all rats were orally challenged with 100 μl of an 18-h culture of *S. mutans* 6715 (4.3×10^6 to 5.2×10^6 CFU) with the aid of a micropipetter. Cages were changed twice daily after each oral immunization to help insure that the antigen in the feces would not be reintroduced into the oral cavity by coprophagy.

At the age of 49 days, rats were removed from the isolators, individual saliva and serum samples were collected (22), and the mandibles were aseptically removed for the quantitation of plaque, levels of *S. mutans* in plaque, and caries scores (26). Briefly, rats were weighed and injected with pentobarbital sodium (23, 26), and saliva was collected with the aid of a capillary pipette after pilocarpine stimulation (23). Whole saliva was collected from each rat over a 20- to 30-min interval, centrifuged (2,800 × *g*, 15 min), and stored at −20°C until assayed for antibody activity (see below). Rats were then bled by cardiac puncture, and individual blood samples were allowed to clot at room temperature followed by overnight incubation at 4°C. Serum was collected after centrifugation (4,400 × *g*, 15 min) and stored at −20°C until assayed. The mandibles from individual rats were aseptically removed and defleshed. The left mandible from each rat was stained with safranin and scored for plaque (26). The right mandible was transferred to a tube containing 3 ml of 0.067 M sterile phosphate buffer, pH 7.0. The plaque was disrupted from the molar surfaces by sonication (Sonifier cell disruptor; Branson Instruments Co., Plain View, N.Y.), and the number of *S. mutans* (and the absence of contaminating microorganisms) in the plaque was determined after the cultivation of samples on blood and mitis salivarius agar (26). The mandibles were then stained with murexide (0.04% ethanol) and hemisectioned, and buccal, sul-

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

Experimental group	Level of anti-*S. mutans* antibodies (EU)[a] in:				
	Saliva		Serum		
	IgA	IgG	IgM	IgG	IgA
Infected only	<5.0	<5.0	<5.0	<5.0	<5.0
S. mutans 6715 WC only	43.6 ± 2.1	11.9 ± 0.6	<5.0	6.6 ± 0.9	22.0 ± 0.6
S. mutans 6715 WC + ConA at:					
100 μg	44.2 ± 2.3	11.6 ± 0.7	<5.0	6.4 ± 0.5	21.7 ± 0.8
500 μg	56.2 ± 3.8	14.1 ± 0.8	7.3 ± 0.6	9.9 ± 0.8	27.1 ± 0.7
2 mg	26.1 ± 0.9	6.8 ± 0.5	<5.0	<5.0	7.9 ± 0.9
ConA only (2 mg)	<5.0	<5.0	<5.0	<5.0	<5.0

[a] As determined by ELISA with optimal concentrations of anti-rat μ-, γ-, or α-heavy-chain-specific sera. Values are expressed as ELISA units (EU), where EU = the mean reciprocal of the dilution of triplicate tests per sample per group (12 to 15 rats per group) giving an optical density reading at 405 nm of 0.1 after 1.5 h of incubation with a substrate.

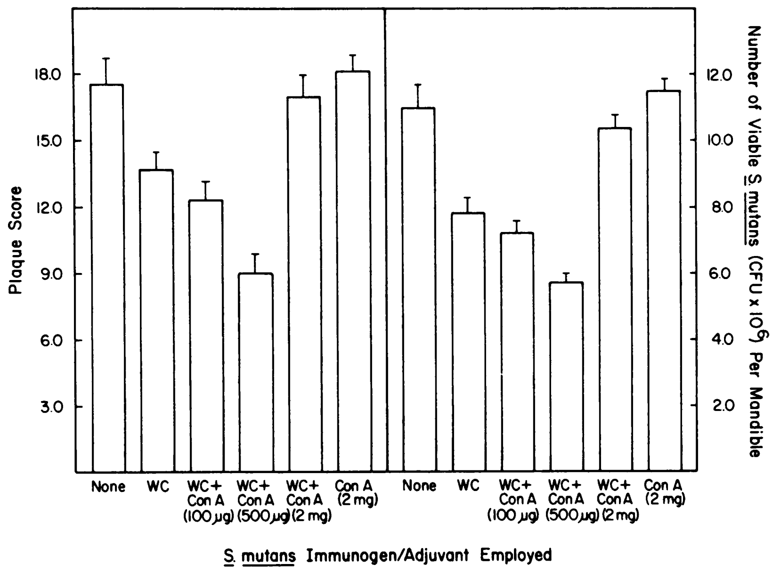

FIG. 2. Plaque scores and numbers of viable *S. mutans* in plaque of gnotobiotic rats (age, 49 days) infected with *S. mutans* 6715 only (none; 13 rats) or given either *S. mutans* 6715 WC (15 rats), WC plus 100 μg of ConA (12 rats), WC plus 500 μg of ConA (12 rats), WC plus 2 mg of ConA (13 rats), or 2 mg of ConA only (12 rats) by GI and infected at the age of 25 days with viable *S. mutans* 6715. The values are the mean ± the standard error of the mean.

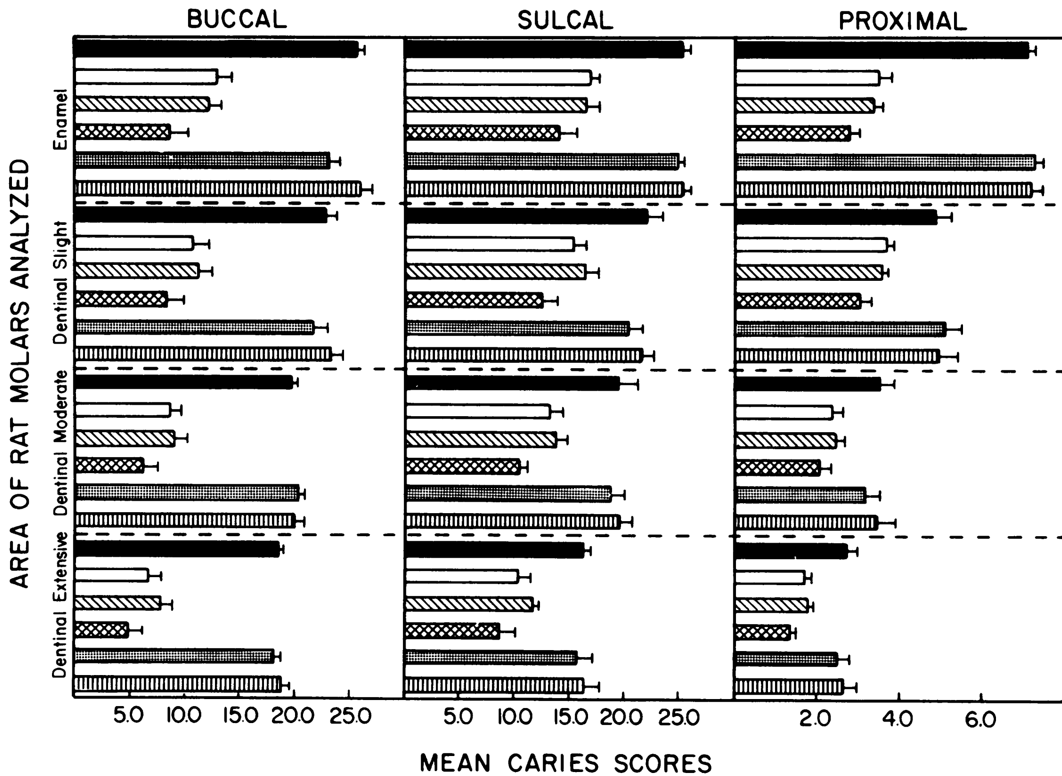

FIG. 3. Caries scores on buccal, sulcal, and proximal molar surfaces of groups of gnotobiotic rats (age, 49 days) infected with *S. mutans* 6715 only (■; 13 rats) or given either *S. mutans* 6715 WC (□; 15 rats), WC plus 100 μg of ConA (▨; 12 rats), WC plus 500 μg of ConA (▩; 12 rats), WC plus 2 mg of ConA (▦; 13 rats), or 2 mg of ConA only (▥; 12 rats) by GI and infected at the age of 25 days with viable *S. mutans* 6715. The values are the mean ± the standard error of the mean.

cal, and proximal molar caries were scored by the Keyes procedure as adapted by our laboratory (26).

Antibody assay. The isotype, level, and specificity of antibodies in saliva and serum samples from the experimental animals were determined by an enzyme-linked immunosorbent assay (ELISA) (7, 40). An antigen suspension or solution (200 μl/well) in 0.1 M carbonate buffer (pH 9.6) was added to the wells of flat-bottom polystyrene microtiter plates (Linbro; Flow Laboratories, Inc., McLean, Va.). The plates were incubated overnight at 37°C, and then the unbound antigen was removed by repeated washing with saline containing 0.05% Tween 20 (Tween-saline). After the final wash, 1% bovine serum albumin in Tween-saline (200 μl/well) was added to individual wells and incubated at 25°C for 90 min. The plates were extensively washed, and appropriate dilutions of either saliva or serum were added (100 μl/well). After incubation (25°C, 3 h), the plates were washed (three times), and alkaline phosphatase-labeled rabbit anti-rat μ-, γ-, or α-heavy-chain-specific antibody was added to appropriate wells (22, 40). The plates were incubated overnight (4°C) and washed, and *p*-nitrophenylphosphate substrate (104 phosphatase substrate; Sigma) dissolved in diethanolamine buffer (pH 9.8, 1 mg/ml) was added (100 μg/well). The amount of color that developed after incubation (25°C, 1.5 h) was measured at 405 nm in the microtiter plate with a Titertek Multiskan photometer (Flow).

Purified *S. mutans* CW components. *S. mutans* WC, CW (described above), serotype *g* carbohydrate, LTA, and dextran were employed as the coating antigens for the assessment of antibody levels and specificity by ELISA. Serotype *g* carbohydrate was prepared from lyophilized *S. mutans* 6715 WC by a modified Rantz-Randall method. Briefly, *S. mutans* 6715 (10 g [dry weight]) was suspended in 200 ml of saline and autoclaved (121°C) for 30 min. The cells were removed by centrifugation, the supernatant was removed and saved, and the cell sediment was subjected to this same treatment two additional times. The 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. 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*.

LTA was prepared by a modification of the phenol-water extraction method of Moskowitz (29). Briefly, *Streptococcus pyogenes* MJM14 WC (400 mg) were suspended in distilled water (20 ml), followed by the addition of an equal volume of 90% phenol. After incubation at 25°C for 1 h with occasional shaking, the water phase was removed, and the extraction procedure was repeated by the addition of 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. Dextran T-2000 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) was further purified by fractionation on Sepharose 2B, and the peak 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 the means and standard errors. The differences among means were evaluated by an analysis of variance and by multiple mean comparisons by the Duncan test. The results of the plaque scores and the number of *S. mutans* colony-forming units per rat mandible were expressed as the mean ± the standard error. The significance of difference between means was determined by Student's *t* test.

RESULTS

Effect of ConA on immune responses to orally administered *S. mutans* antigen. Our past studies

TABLE 2. Levels of anti-*S. mutans* 6715 antibodies in serum and saliva of gnotobiotic rats given either *S. mutans* 6715 WC or CW and MDP by GI

Experimental group	Level of anti-*S. mutans* antibodies (EU)[a] in:				
	Saliva		Serum		
	IgA	IgG	IgM	IgG	IgA
Infected only	<5.0	<5.0	<5.0	<5.0	<5.0
S. mutans 6715 WC only	41.7 ± 1.8	11.6 ± 0.9	<5.0	6.8 ± 0.6	21.6 ± 0.8
S. mutans 6715 WC + MDP at:					
62.5 μg	59.8 ± 2.4	20.6 ± 0.6	8.7 ± 0.9	11.2 ± 0.6	31.6 ± 1.1
250 μg	89.7 ± 3.6	26.9 ± 0.9	11.2 ± 0.7	14.1 ± 0.7	38.8 ± 1.4
S. mutans 6715 CW only	40.6 ± 1.3	10.8 ± 0.8	<5.0	6.1 ± 0.5	18.7 ± 0.5
S. mutans 6715 CW + MDP at:					
62.5 μg	54.5 ± 1.6	18.7 ± 0.9	6.4 ± 0.6	8.9 ± 0.7	24.6 ± 0.7
250 μg	89.6 ± 3.2	25.8 ± 1.0	10.7 ± 0.5	18.4 ± 0.9	29.9 ± 0.9
MDP only (250 μg)	<5.0	<5.0	<5.0	<5.0	<5.0

[a] As determined by ELISA with optimal concentrations of anti-rat μ-, γ-, or α-heavy-chain-specific sera. Values are expressed as ELISA units (EU), where EU = the mean reciprocal of the dilution of triplicate tests per sample per group (12 to 17 rats per group) giving an optical density reading at 405 nm of 0.1 after 1.5 h of incubation with a substrate.

TABLE 3. Specificity of salivary and serum IgA anti-*S. mutans* 6715 antibodies of gnotobiotic rats given either *S. mutans* 6715 WC or CW and MDP by GI

Experimental group	Level of anti-*S. mutans* antibodies (EU)[a] in: Salivary IgA					Serum IgA				
	WC	CW	LTA	RR *g*	Dextran	WC	CW	LTA	RR *g*	Dextran
Infected only	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0
S. mutans 6715 WC only	42.4 ± 2.1	34.4 ± 1.6	38.8 ± 1.9	18.4 ± 0.7	17.4 ± 0.6	22.3 ± 1.4	17.1 ± 0.9	20.4 ± 0.9	9.6 ± 0.6	8.8 ± 0.5
S. mutans 6715 + MDP (250 μg)	89.7 ± 3.2	68.8 ± 2.4	101.7 ± 4.4	39.8 ± 1.5	27.3 ± 1.3	36.7 ± 1.9	31.6 ± 1.1	39.8 ± 1.8	14.6 ± 0.5	12.7 ± 0.7
S. mutans 6715 CW only	39.8 ± 1.5	43.4 ± 2.3	14.4 ± 0.6	28.6 ± 1.8	24.7 ± 1.1	18.4 ± 1.7	22.8 ± 1.1	<5.0	15.0 ± 0.6	12.4 ± 0.5
S. mutans 6715 CW + MDP (250 μg)	79.6 ± 3.1	89.4 ± 3.6	26.7 ± 0.9	51.4 ± 2.4	41.2 ± 1.9	31.4 ± 2.1	38.9 ± 2.2	7.1 ± 0.4	26.1 ± 1.1	20.0 ± 0.9
MDP only (250 μg)	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0

[a] As determined by ELISA with optimal concentrations of anti-rat α-heavy-chain-specific sera. Values are expressed as ELISA units (EU), where EU = the mean reciprocal of the dilution of quadruplicate tests per sample per group (12 to 17 rats per group) giving an optical density reading at 405 nm of 0.1 after 1.5 h of incubation with a substrate.

with LPS-nonresponsive C3H/HeJ mice indicated that the GI of thymic-dependent antigen induced greater T helper cell activity in GALT, i.e., PP, than that seen in identically treated LPS-responsive C3H/HeN mice (11). Furthermore, the oral administration of ConA with antigen resulted in increased T helper cell activity in PP and elevated IgA responses (11–13). Therefore, in our first series of experiments, we evaluated the ability of ConA to enhance the humoral immune responses of rats, when given 4 h before *S. mutans* 6715 WC by GI. A slight increase in salivary and serum IgA levels was noted in gnotobiotic rats given *S. mutans* 6715 WC and 500 μg of ConA (Table 1); higher levels of ConA (2 mg) actually depressed the responses when compared with the response obtained in rats given antigen alone. These results suggest that ConA is not a good oral adjuvant in gnotobiotic Fischer rats and, depending upon the dose of ConA employed, may actually suppress the response to the antigen given by the oral route.

The number of viable *S. mutans* in plaque and caries scores from rats given antigen and ConA reflected the antibody response obtained in these animals (Fig. 2 and 3). Rats receiving *S. mutans* 6715 WC were protected against challenge with virulent *S. mutans* when compared with the infected controls. Although animals receiving *S. mutans* 6715 WC and 500 μg of ConA exhibited slightly lower plaque scores and levels of viable *S. mutans* in plaque (Fig. 2) and caries activity (Fig. 3) than did rats immunized with antigen alone, the difference was not significant ($P \leq 0.05$). Of interest was the observation that rats receiving the highest dose of ConA, which exhibited depressed levels of salivary and serum antibody responses when compared with animals given antigen alone, were not protected against infection with virulent *S. mutans* 6715. Although other explanations for this lack of protection could be provided, it is tempting to suggest that the lower IgA response observed in these rats was insufficient for protection against the *S. mutans* challenge.

Enhancement by oral MDP of immune responses to *S. mutans* particulate antigen and protection. In the next series of experiments, we evaluated the adjuvant peptide MDP for its ability to potentiate the immune response to particulate antigens of *S. mutans* 6715 given by the oral route. In these studies, we used both *S. mutans* 6715 WC and purified CW as the oral immunogens, since we have previously shown that the GI of these antigen forms induces immune responses, especially salivary IgA responses, and subsequent protection against caries formation (27). Both *S. mutans* 6715 WC and CW given by GI induced similar salivary IgA responses (Table 2); serum IgA responses were also noted.

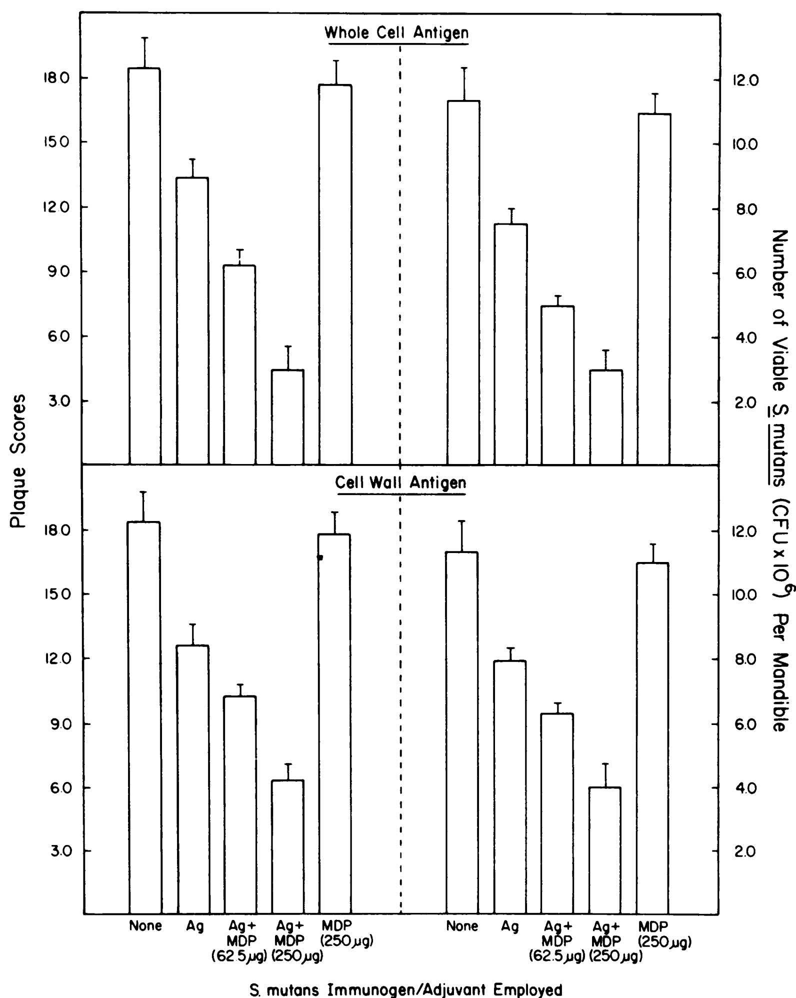

FIG. 4. Plaque scores and number of viable *S. mutans* in plaque of gnotobiotic rats (age, 49 days) infected with *S. mutans* 6715 only (none; 12 rats) or given either *S. mutans* 6715 WC (Ag; 12 rats), WC plus 62.5 μg of MDP (15 rats), WC plus 250 μg of MDP (17 rats), *S. mutans* 6715 CW (15 rats), CW plus 62.5 μg of MDP (16 rats), CW plus 250 μg of MDP (15 rats), or 250 μg of MDP only (12 rats) by GI and infected at the age of 25 days with viable *S. mutans* 6715. The values are the mean ± the standard error of the mean.

Rats given MDP together with the particulate antigen exhibited significantly ($P \leq 0.05$) higher salivary and serum immune responses than did animals given the antigen alone. Although an MDP dose of 62.5 μg enhanced antibody responses to *S. mutans* antigens, the maximum augmentation of immune responses was obtained when a dose of 250 μg of MDP was given orally with the antigen (Table 2). Higher doses of MDP (e.g., 1 mg per rat) did not potentiate the immune response to the antigen to a greater degree than did a dose of 250 μg of the adjuvant (data not shown). It should be noted that both major immunoglobulin isotypes in saliva, i.e., IgA and IgG, were significantly ($P \leq 0.05$) enhanced by MDP. This enhancement was not due to polyclonal activation, since no serum or salivary anti-*S. mutans* antibody responses were detected in rats given MDP alone by GI.

When the specificity of salivary and serum IgA antibodies to individual *S. mutans* CW determinants was ascertained, an interesting pattern emerged (Table 3). Rats given *S. mutans* 6715 WC and MDP (250 μg) exhibited significantly ($P \leq 0.05$) higher levels of salivary IgA antibodies to WC, CW, LTA, and to serotype carbohydrate (RR *g*) than did rats given antigen alone. Although serum IgA antibody levels were lower than those of saliva, a similar pattern of antibody specificity was observed. These data suggest that the major response to orally administered *S. mutans* antigen is of the IgA isotype

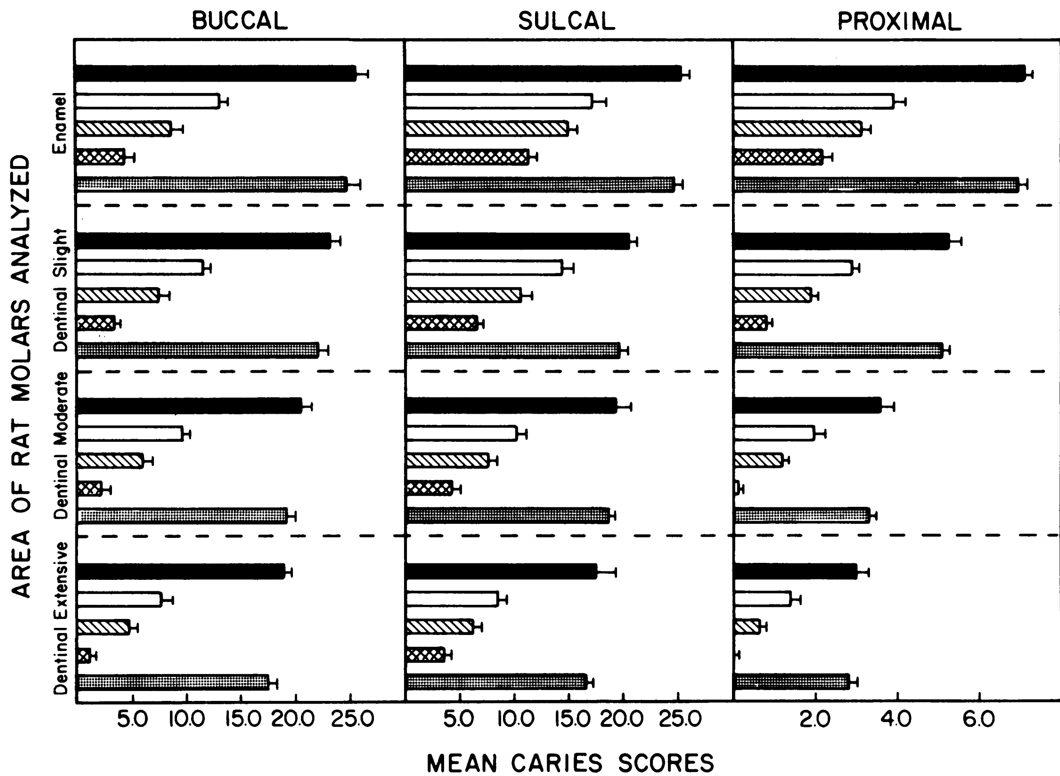

FIG. 5. Caries scores on buccal, sulcal, and proximal molar surfaces of groups of gnotobiotic rats (age, 49 days) infected with *S. mutans* 6715 only (■; 12 rats) or given either *S. mutans* 6715 WC (□; 12 rats), WC plus 62.5 μg of MDP (▨; 15 rats), WC plus 250 μg of MDP (▩; 17 rats), or 250 μg of MDP only (▤; 12 rats) by GI and infected at the age of 25 days with viable *S. mutans* 6715. The values are the mean ± the standard error of the mean.

(Table 2) and that the individual specificities of the IgA antibodies are similar in serum and saliva (Table 3).

When purified *S. mutans* 6715 CW was used as the immunogen and MDP (250 μg) as the adjuvant, we found slightly higher levels of salivary IgA antibodies to purified CW than were demonstrated in animals given *S. mutans* 6715 WC antigen only by GI (Table 3). This was not surprising and probably reflects a better display of antigens in CW for the measurement of specific antibodies to CW components than with WC. This point is well illustrated by the demonstrated levels of specific antibody to LTA and RR *g* in the saliva of rats given either *S. mutans* 6715 WC or CW antigen. It is known that LTA is present in much lower quantities in CW than in WC and, indeed, lower levels of anti-LTA antibodies were induced with CW than with WC (Table 3). On the other hand, purified CW contains greater amounts of serotype carbohydrate on a weight basis than do WC, and animals immunized with CW and MDP exhibited higher levels of antibodies to RR *g* than did rats given WC antigen and MDP. A similar pattern of response was seen when serum antibody specificities were determined in these groups of rats. No polyclonal responses to individual cell surface determinants were seen when MDP was administered without antigen to rats (Table 3).

Since high salivary immune responses were induced in rats given *S. mutans* 6715 WC or CW and MDP, it was of interest next to determine whether this response altered the colonization pattern of *S. mutans* and caries protection (Fig. 4–6). Significantly ($P < 0.05$) lower levels of plaque were seen on mandibular molars from rats given *S. mutans* 6715 WC or CW and 250 μg of MDP when compared with infected-only rats given the oral vaccine without the adjuvant (Fig. 4). The reduced plaque scores correlated directly with the level of viable *S. mutans* 6715 in plaque, since rats receiving particulate antigen and 250 μg of MDP exhibited significantly ($P \leq 0.05$) fewer viable *S. mutans* in plaque than did rats only infected with *S. mutans* or animals given the particulate antigen vaccine alone (Fig. 4).

The caries scores in the various groups of rats reflected the magnitude of the salivary antibody

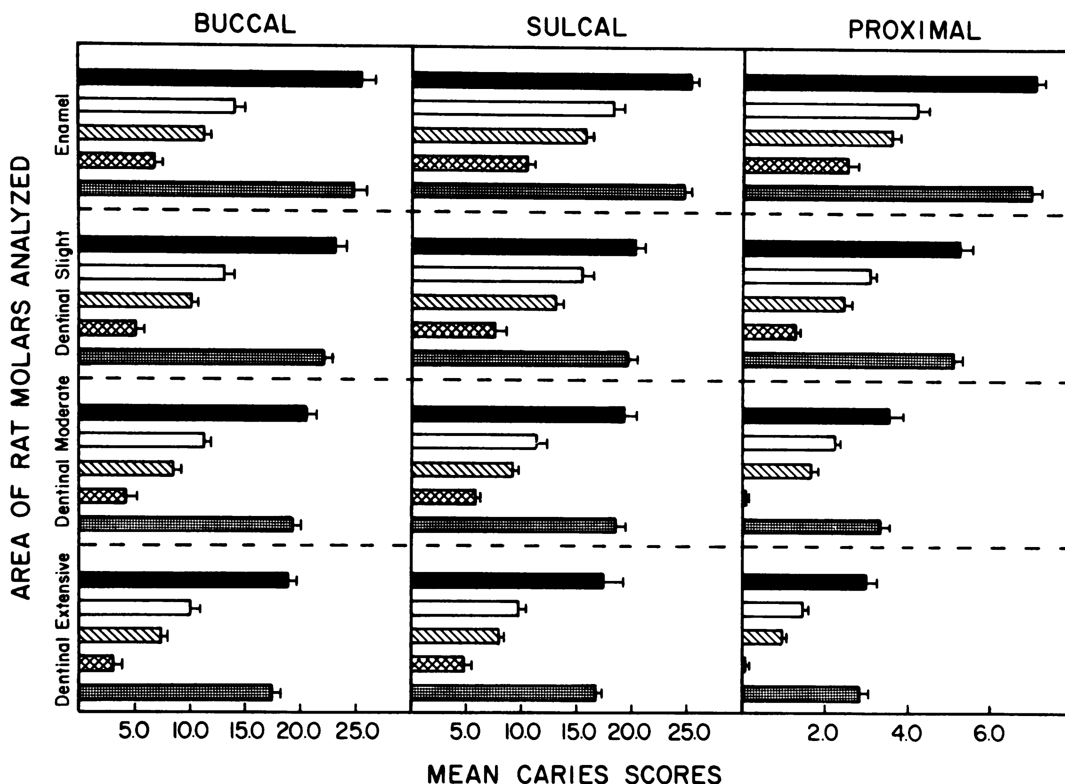

FIG. 6. Caries scores on buccal, sulcal, and proximal molar surfaces of groups of gnotobiotic rats (age, 49 days) infected with *S. mutans* 6715 only (■; 12 rats) or given either *S. mutans* 6715 CW (□; 15 rats), CW plus 62.5 μg of MDP (▨; 16 rats), CW plus 250 μg of MDP (▩; 15 rats), or 250 μg of MDP only (▦; 12 rats) by GI and infected at the age of 25 days with viable *S. mutans* 6715. The values are the mean ± the standard error of the mean.

response to the antigen (Fig. 5 and 6). Rats receiving particulate *S. mutans* vaccine, either WC (Fig. 5) or CW (Fig. 6), exhibited significantly ($P \leq 0.05$) lower caries scores on all molar surfaces when compared with rats only infected with *S. mutans* 6715. Interestingly, the protection observed was similar for the two antigen forms (i.e., WC or CW); however, the

TABLE 4. Levels of anti-*S. mutans* 6715 antibodies in serum and saliva of gnotobiotic rats given *S. mutans* 6715 WC or CW and PG by GI

Experimental group	Level of anti-*S. mutans* antibodies (EU)[a] in:				
	Saliva		Serum		
	IgA	IgG	IgM	IgG	IgA
Infected only	<5.0	<5.0	<5.0	<5.0	<5.0
S. mutans 6715 WC only	38.9 ± 2.1	12.8 ± 0.6	<5.0	9.5 ± 0.4	18.4 ± 0.8
S. mutans 6715 WC + PG at:					
62.5 μg	55.9 ± 3.2	18.5 ± 0.5	8.2 ± 0.4	12.1 ± 0.5	21.8 ± 0.7
250 μg	76.2 ± 4.5	21.6 ± 0.9	12.1 ± 0.5	15.8 ± 0.5	23.6 ± 0.9
S. mutans 6715 CW only	38.9 ± 1.7	11.3 ± 0.5	<5.0	6.9 ± 0.4	17.9 ± 0.7
S. mutans 6715 CW + PG at:					
62.5 μg	58.3 ± 2.9	19.6 ± 0.8	9.1 ± 0.5	11.8 ± 0.4	20.1 ± 0.8
250 μg	85.6 ± 4.9	26.1 ± 1.1	10.9 ± 0.4	26.7 ± 1.2	29.3 ± 1.1
PG only (250 μg)	<5.0	6.1 ± 0.3	<5.0	11.3 ± 0.4	<5.0

[a] As determined by ELISA with optimal concentrations of anti-rat μ-, γ-, or α-heavy-chain-specific sera. Values are expressed as ELISA units (EU), where EU = the mean reciprocal of the dilution of triplicate tests per sample per group (13 to 18 rats per group) giving an optical density reading at 405 nm of 0.1 after 1.5 h of incubation with a substrate.

TABLE 5. Specificity of salivary and serum IgA anti-*S. mutans* 6715 antibodies of gnotobiotic rats given either *S. mutans* 6715 WC or CW and PG by GI

Experimental group	Level of anti-*S. mutans* antibodies (EU)[a]									
	Salivary IgA					Serum IgA				
	WC	CW	LTA	RR g	Dextran	WC	CW	LTA	RR g	Dextran
Infected only	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0	<5.0
S. mutans 6715 WC only	44.6 ± 2.1	32.5 ± 1.9	39.9 ± 2.1	22.1 ± 1.1	18.3 ± 0.9	20.0 ± 1.0	17.6 ± 0.9	23.6 ± 1.3	8.9 ± 0.6	6.7 ± 0.5
S. mutans 6715 + PG (250 μg)	78.2 ± 3.7	69.9 ± 3.1	74.3 ± 3.6	41.3 ± 2.8	25.8 ± 1.5	36.6 ± 2.0	35.0 ± 2.1	44.0 ± 2.6	15.2 ± 0.7	12.4 ± 0.8
S. mutans 6715 CW only	37.6 ± 1.8	40.1 ± 2.2	14.6 ± 0.6	25.0 ± 0.9	26.9 ± 1.1	18.2 ± 0.9	23.6 ± 1.3	<5.0	17.1 ± 0.8	16.2 ± 0.7
S. mutans 6715 + PG (250 μg)	89.2 ± 4.3	93.4 ± 5.4	29.1 ± 1.1	54.1 ± 2.9	53.3 ± 2.1	30.4 ± 1.6	48.1 ± 1.9	<5.0	34.9 ± 1.5	30.0 ± 1.6
PG only (250 μg)	6.1 ± 0.3	<5.0	6.4 ± 0.3	<5.0	<5.0	<5.0	<5.0	6.1 ± 0.4	<5.0	<5.0

[a] As determined by ELISA with optimal concentrations of anti-rat α-heavy-chain-specific sera. Values are expressed as ELISA units (EU), where EU = the mean reciprocal of the dilution of quadruplicate tests per sample per group (13 to 18 rats per group) giving an optical density reading of 0.1 after 1.5 h of incubation with a substrate.

former vaccine resulted in slightly better protection (Fig. 5). On the other hand, when MDP was administered with either of these antigen forms, a marked reduction in caries scores was seen (Fig. 5 and 6). Although complete protection was not observed in groups of rats given the antigen and 250 μg of MDP, the level of caries on all molar surfaces was significantly ($P \leq 0.05$) lower than that obtained in rats given the antigen alone.

Potentiation by *S. mutans* PG of immune responses to orally administered antigen. The demonstration that MDP is an effective oral adjuvant capable of enhancing salivary responses to *S. mutans* and caries protection is promising and suggests that the basic adjuvant component of complete Freund adjuvant effectively promotes immune responses to orally administered antigens. Since PG is a major component of the *S. mutans* CW, we considered the possibility that adjuvant derivatives from this structure could be used in an oral vaccine against dental caries. Our previous studies (14) have indicated that the oral administration of PG with haptenated *S. mutans* to mice enhanced splenic plaque-forming-cell responses, especially of the IgA isotype. Therefore, in the next series of experiments, gnotobiotic rats were given *S. mutans* 6715 WC or purified CW either alone or together with purified *S. mutans* PG by GI. We measured the levels and isotype of anti-*S. mutans* antibodies in serum and saliva and found that the GI of rats with PG and antigen resulted in augmented immune responses (Table 4). Both salivary IgA and IgG responses were enhanced approximately twofold by the oral administration of 250 μg of PG with either WC or CW immunogen. Higher doses of PG did not enhance the response above that seen with 250 μg of PG; however, they did result in increased polyclonal responses (data not shown). A slight polyclonal IgG response was noted in rats given 250 μg of PG (Table 4).

A comparison of the specificities of salivary and serum IgA antibodies from rats given either *S. mutans* 6715 WC or CW and *S. mutans* PG (250 μg) revealed a pattern similar to that obtained when MDP was used as the oral adjuvant (Tables 3 and 5). When *S. mutans* 6715 WC was used as the immunogen, we found good serum and salivary IgA antibody levels to LTA, which were significantly ($P \leq 0.05$) enhanced by PG (Table 5). On the other hand, *S. mutans* 6715 CW and PG induced low anti-LTA levels in saliva and none in serum. *S. mutans* 6715 CW induced high anti-carbohydrate responses, which were enhanced approximately twofold by PG.

When the levels of plaque and numbers of viable *S. mutans* in plaque were assessed, rats given *S. mutans* antigen and 250 μg of PG

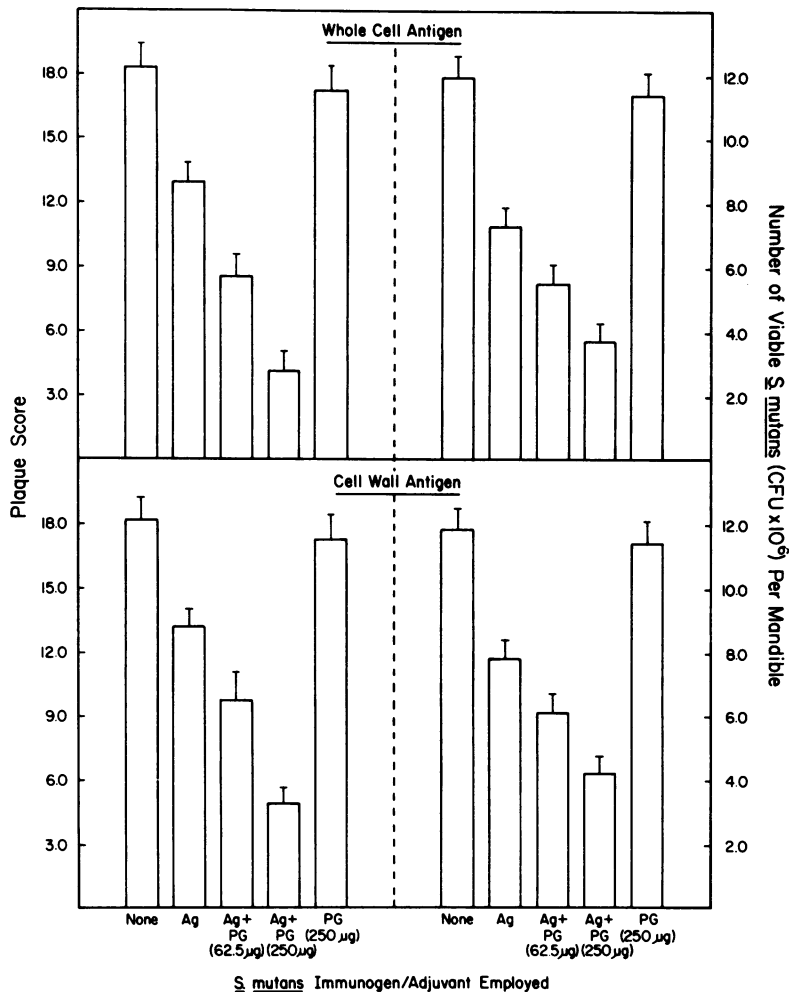

FIG. 7. Plaque scores and number of viable *S. mutans* in plaque of gnotobiotic rats (age, 49 days) infected with *S. mutans* 6715 only (none; 13 rats) or given either *S. mutans* 6715 WC (Ag; 14 rats), WC plus 62.5 μg of PG (15 rats), WC plus 250 μg of PG (18 rats), *S. mutans* 6715 CW (12 rats), CW plus 62.5 μg of PG (17 rats), CW plus 250 μg of PG (16 rats), or 250 μg of PG only (13 rats) by GI and infected at the age of 25 days with viable *S. mutans* 6715. The values are the mean ± the standard error of the mean.

exhibited significantly ($P \leq 0.05$) lower plaque scores and numbers of viable *S. mutans* in plaque when compared with animals given the antigen alone or infected-only rats (Fig. 7). Furthermore, reduced levels of viable *S. mutans* in plaque directly correlated with reduced caries scores in these rats (Fig. 8 and 9). Rats receiving either *S. mutans* 6715 WC (Fig. 8) or CW (Fig. 9) and 250 μg of PG exhibited significantly ($P \leq 0.05$) lower caries scores on all molar surfaces when compared with rats given the antigen alone.

DISCUSSION

It is now accepted that the oral administration of either replicating antigens (30, 31) or particulate antigen forms (21, 24, 25, 28) induces IgA immune responses in several external secretions, and evidence for a common mucosal immune defense system has been reviewed previously (21). However, the oral administration of some antigens is ineffective for the induction of mucosal immune responses, and it is therefore important to evaluate suitable adjuvants which may be administered by the oral route and enhance the production of antibodies in various external secretions. We have previously shown that the provision of gnotobiotic rats with *S. mutans* WC in their drinking water or food results in IgA responses in saliva and caries protection (21, 24, 25). We have recently provided evidence that the administration of *S. mutans*

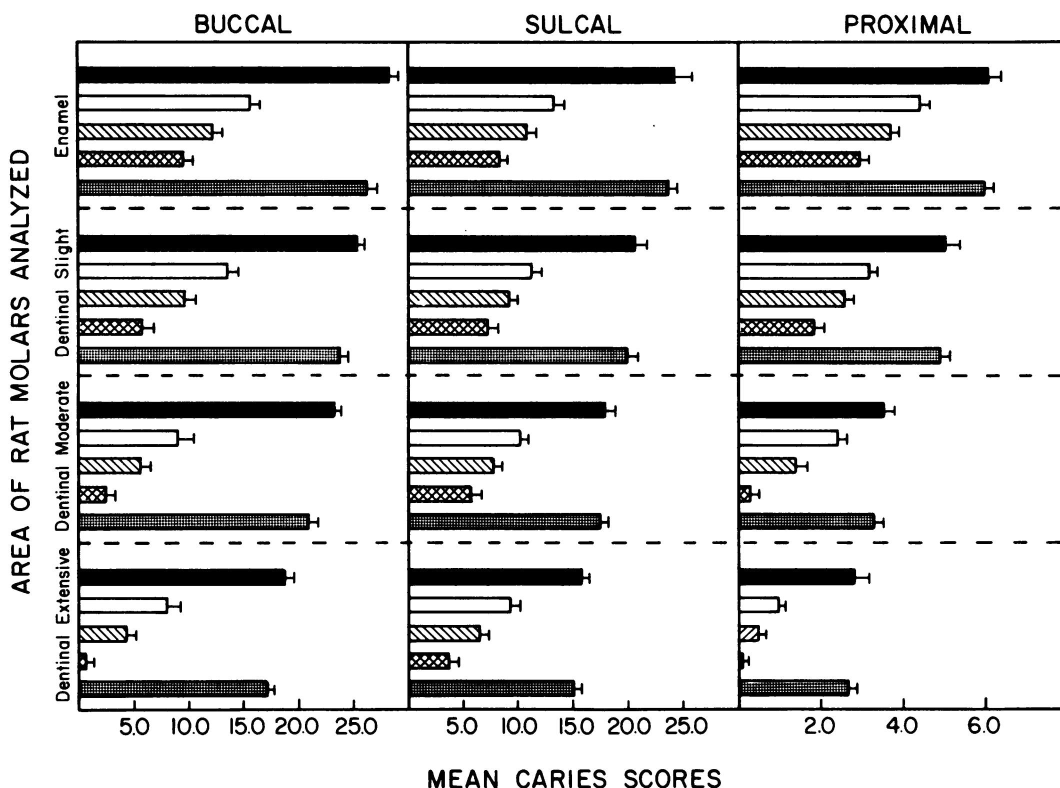

FIG. 8. Caries scores on buccal, sulcal, and proximal molar surfaces of groups of gnotobiotic rats (age, 49 days) infected with *S. mutans* 6715 only (■; 13 rats) or given either *S. mutans* 6715 WC (□; 14 rats), WC plus 62.5 μg of PG (▨; 15 rats), WC plus 250 μg of PG (▩; 18 rats), or 250 μg of PG only (▦; 13 rats) by GI and infected at the age of 25 days with viable *S. mutans* 6715. The values are the mean ± the standard error of the mean.

WC or CW by gavage also results in salivary IgA responses and caries protection (27), and the results of the present study confirm this finding. Since complete protection against challenge with a virulent *S. mutans* is difficult to attain, it is of considerable importance to attempt to potentiate salivary IgA responses which would result in better caries protection.

In the study reported here, we have shown that the oral administration of particulate *S. mutans* antigens with either MDP or *S. mutans* CW-derived PG results in enhanced salivary IgA anti-*S. mutans* antibody levels. This finding was not totally unexpected since we have previously shown that the oral administration of trinitrophenyl (TNP)-haptenated *S. mutans* and either MDP or PG to mice results in enhanced anti-TNP plaque-forming-cell and antibody responses (14). Furthermore, it is well known that MDP exhibits adjuvant properties for a number of antigens when administered by the oral route (3). Recent studies by Taubman and co-workers (38) have shown that the GI of *S. mutans* GTF and MDP to rats results in higher salivary IgA responses than those seen in animals given GTF only. The present study represents the first demonstration that orally administered antigen, together with MDP or PG, enhances the level of salivary antibodies, most notably of the IgA isotype, and that this increased antibody level directly correlates with a greater reduction of *S. mutans* in plaque and caries immunity.

Our past work with lymphoid cells derived from murine PP suggested that MDP potentiates responses via direct effects on the PP macrophage (12). This was primarily based upon the observation that LPS-nonresponsive C3H/HeJ macrophages were unresponsive to MDP in vitro, whereas enhanced in vitro responses of C3H/HeN PP cell cultures to thymic-dependent antigens were obtained. Chedid and his colleagues (8, 20) and Kotani and co-workers (32) have also presented evidence that MDP potentiates responses by a direct effect on macrophages. Therefore, it is tempting to postulate that the oral administration of particulate *S. mutans* antigen with MDP potentiates the induction of the IgA response directly in the PP.

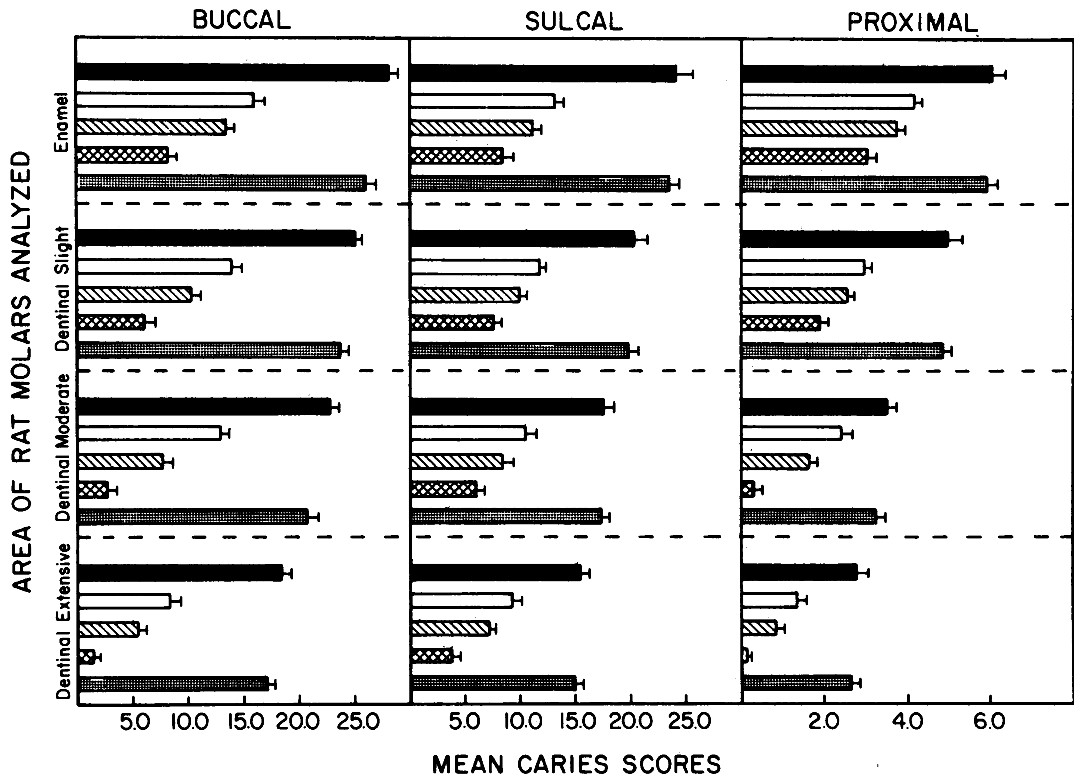

FIG. 9. Caries scores on buccal, sulcal, and proximal molar surfaces of groups of gnotobiotic rats (age, 49 days) infected with *S. mutans* 6715 only (■; 13 rats) or given either *S. mutans* 6715 CW (□; 12 rats), CW plus 62.5 μg of PG (▨; 17 rats), CW plus 250 μg of PG (▩; 16 rats), or 250 μg of PG only (▦; 13 rats) by GI and infected at the age of 25 days with viable *S. mutans* 6715. The values are the mean ± the standard error of the mean.

However, we cannot distinguish this possibility from other mechanisms for MDP activity, since it is likely that this low-molecular-weight compound reaches systemic tissue and perhaps induces stimulatory effects in these sites. In this regard, MDP was effective in potentiating both serum and salivary IgA anti-*S. mutans* responses.

Our results with *S. mutans* CW PG indicate that the *S. mutans* CW possesses innate adjuvant properties. We cannot as yet ascribe the adjuvant properties of PG solely to the presence of muramyl peptide derivatives, although a significant portion of PG is represented by muramic acid peptide moieties. It is possible that PG also contains other immunostimulatory activity, since bacterial PG is a good B cell mitogen and polyclonal activator (5, 6). On the other hand, purified MDP and synthetic analogs of this compound are poor mitogens and polyclonal B cell activators (4, 41). Therefore, it will be necessary to further purify the PG components of *S. mutans* CW to determine the individual components responsible for the adjuvant properties. Studies along these lines are currently in progress.

The finding that ConA was a poor oral adjuvant in Fischer rats given *S. mutans* particulate antigen by gavage was somewhat surprising. It has been reported previously (9) that ConA binds to the surface of *S. mutans* and effectively aggregates the cells. We have also observed the aggregation of *S. mutans* 6715 WC by ConA. Although in the present study, we administered ConA by gavage before *S. mutans* 6715 WC antigen, it is still possible that in vivo agglutination occurred and resulted in less effective antigen presentation to GALT. This effect could have accounted for the depressed immune responses observed with the highest dose of ConA employed with *S. mutans* 6715 WC antigen. An alternate explanation would be that the GI of high doses of ConA selectively stimulates the maturation of T suppressor cells in GALT in Fischer rats, which effectively diminishes the induction of immune responses to orally administered *S. mutans* antigen.

The oral administration of either *S. mutans*

6715 WC or purified CW induced a characteristic pattern of IgA antibody specificities in both serum and saliva. The *S. mutans* 6715 WC vaccine induced good levels of IgA antibodies which were detectable with either WC or CW coating antigens and also resulted in significant levels of IgA anti-LTA antibodies in both saliva and serum. In addition, moderate levels of antibodies of *S. mutans* carbohydrates were induced with this vaccine. Both MDP and PG enhanced the level of these IgA antibodies in both serum and saliva approximately twofold. These results indicate that the specificities of serum and salivary IgA antibodies were similar. The same conclusions were reached when purified CW was used as the immunogen. Since LTA represents only a minor portion of this antigen, low salivary and serum IgA anti-LTA antibody levels were seen. On the other hand, significantly higher levels of IgA anti-carbohydrate antibodies were induced with this vaccine and also with MDP or PG.

The fact that rats receiving purified CW and adjuvants exhibited similar degrees of protection to that seen with WC and an adjuvant could be taken as evidence that IgA anti-LTA antibodies are of less importance in caries immunity. However, this may represent a fallacious argument since more than one cell surface antigen may be of importance in the induction of caries immunity. Thus, higher levels of anti-LTA antibodies may be protective on the one hand, whereas significant salivary responses to carbohydrate antigens or to proteins, such as GTF, may also effect caries immunity. The net result of IgA antibodies to *S. mutans* surface determinants might result in similar degrees of caries immunity, although the mechanisms of antibody activity may be completely different. Thus, studies of caries immunity must continue to carefully evaluate antigen form and the specificity of antibodies induced in saliva to antigens in different states of purity.

In summary, we have presented evidence that the oral administration of soluble adjuvants, namely, MDP and *S. mutans* PG, potentiates immune responses in saliva and serum to particulate antigen forms of *S. mutans*. These adjuvants resulted in caries scores which were significantly lower than those obtained with the oral antigen alone. These studies should now allow us to determine the precise mechanisms whereby MDP and other derivatives of PG enhance the immune responses to orally administered antigen.

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