INFECTION AND IMMUNITY, Oct. 1990, p. 3407–3414
0019-9567/90/103407-08$02.00/0

Vol. 58, No. 10

An Investigation into the Mechanism of Protection by Local Passive Immunization with Monoclonal Antibodies against *Streptococcus mutans*

JULIAN K.-C. MA,* MANJEET HUNJAN, ROBERTA SMITH, CHARLES KELLY, AND THOMAS LEHNER

Department of Immunology, United Medical and Dental Schools of Guy's and St. Thomas's Hospitals, Floor 28, Guy's Tower, London Bridge, London SE1 9RT, United Kingdom

Received 21 March 1990/Accepted 2 August 1990

Local oral passive immunization with *Streptococcus mutans*-specific monoclonal antibody (MAb) (Guy's 13) prevented recolonization by indigenous *S. mutans* in human volunteers who had first been treated with a conventional antibacterial agent (chlorhexidine). The $F(ab')_2$ fragment of the MAb was as protective as the intact immunoglobulin G, but the Fab fragment of the molecule failed to prevent recolonization of *S. mutans*. In subjects receiving the MAb Fab fragment, *S. mutans* levels in dental plaque and saliva reappeared at a similar rate to that found in sham-immunized subjects who received either saline or a nonprotective MAb. In vitro, MAb had no bacteriostatic or bacteriocidal effect on *S. mutans*. However, *S. mutans* grown in the presence of either intact immunoglobulin G MAb or the $F(ab')_2$ fragment formed very long chains, which resulted in clumping of the cells. *S. mutans* grown with either saline or the MAb Fab fragment formed significantly shorter chains, more characteristic of streptococcal growth in liquid media. The results suggest that the two binding sites of the MAb molecule may be an essential feature for preventing streptococcal colonization but that the ability to bind to phagocytes and activate complement which resides in the Fc fragment is not essential. Protection against colonization by *S. mutans* lasting up to 2 years was observed in immunized subjects, although MAb was applied over a period of only 3 weeks. Furthermore, functional MAb was detected up to 3 days following application of MAb to the teeth. The long-term protection could not be accounted for by a persistence of MAb on the tooth surface, and we have suggested that it may be due to a shift in the balance of the oral flora which discouraged recolonization by *S. mutans*. However, examination of the proportions of *Streptococcus sanguis* and veillonella species in the recolonization experiments failed to reveal a significant change in the proportions of either organism, which returned to approximately the preexperimental levels in both the immunized and control groups. These findings confirm the in vivo functional specificity of the MAb to *S. mutans* but are not consistent with the suggestion that *S. sanguis* or veillonella take over the niche vacated by *S. mutans*, unless the shift in the proportion of these organisms cannot be detected by the method used.

The bacterium *Streptococcus mutans* is commonly believed to be of importance in the initiation of dental caries (6, 8). A major cell surface determinant is the 185-kilodalton glycoprotein streptococcal antigen (SA) I/II (24–26), which is believed to have a number of important functions, including that of adhesion to the tooth surface (19). Systemic immunization with SA I/II in animal models induces specific serum antibodies (14) which can be expressed on the tooth surface in gingival crevicular fluid (29). A reduction in the levels of *S. mutans* colonization as well as lower caries scores have been demonstrated in rhesus monkeys systemically immunized with SA I/II (14).

Recently, we have proposed local passive immunization as an alternative approach to prevention of colonization of *S. mutans*. Monoclonal antibodies (MAbs) raised against SA I/II applied directly to the teeth prevented colonization by *S. mutans* and protected against caries in two species of nonhuman primates (12, 13). Polyclonal antibodies from hyperimmunized cow milk have also been used to prevent oral colonization by *S. mutans* and dental caries in rodents (20). When used in human volunteers, local passive immunization with MAb significantly reduced implantation of orally administered streptomycin-resistant *S. mutans* (18). MAb also prevented recolonization of indigenous *S. mutans* in subjects whose initial *S. mutans* levels had first been reduced with a topical, broad-spectrum antibacterial agent, chlorhexidine gluconate (17).

In view of the prolonged protection seen in the human studies, an investigation into the possible mode of action of the MAb has been carried out. The aims of the present study were as follows: (i) to determine the duration of MAb in vivo on the tooth surface, (ii) to determine whether the intact immunoglobulin G (IgG) molecule was necessary or whether the $F(ab')_2$ and Fab fragments might be effective in preventing colonization by *S. mutans*, (iii) to determine the effects of local passive immunization with MAb on other oral bacterial strains, and (iv) to examine the effect of MAb on the growth of *S. mutans* in vitro.

MATERIALS AND METHODS

Healthy student volunteers from Guy's Hospital Dental School were used in this investigation. None were taking any medication nor had active caries. The experimental (n = 8) and control subjects (n = 7) were matched for age, sex, caries index (decayed, missing, and filled surfaces), gingival index (16), and plaque index (28). They were allowed to continue with their routine oral hygiene procedures and diet throughout the experimental period, except that they were asked to abstain from eating or drinking for 30 min after every experimental procedure.

MAbs. MAb Guy's 13 is a mouse IgG1 class of antibody

* Corresponding author.

and was originally raised against an equivalent antigen I/II from the cell surface of *Streptococcus sobrinus*. However, it recognizes a common protein epitope shared by other serotypes of mutans streptococci and binds strongly to *S. mutans* (21, 30). The production and purification of the MAb Guy's 13 has been described in detail previously (17, 30). Briefly, an ammonium sulphate precipitate of ascites fluid was suspended in phosphate buffer and treated with DNase and RNase, followed by ultraviolet irradiation. The IgG fraction was recovered by using DEAE cellulose chromatography, dialyzed against distilled water, and freeze-dried. A control MAb, Guy's 11, was prepared in the same way. Although raised against the same antigen as MAb Guy's 13 and sharing a similar cross-reactivity against the other mutans streptococci, MAb Guy's 11 appears to recognize a different epitope to Guy's 13 and was not protective in previous studies in humans (17).

The $F(ab')_2$ fragment of MAb Guy's 13 was prepared by pepsin digestion of the IgG preparation. The lyophilized IgG was suspended at 2 mg/ml in 0.1 M Walpole's acetate buffer (pH 3.8), and pepsin (Sigma Chemical Co., Poole, United Kingdom) was added to a concentration of 1 mg of pepsin to 33 mg of IgG. Digestion was carried out at 37°C for 22 to 24 h, at which point a portion was taken for analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to confirm complete digestion of the IgG. The sample was then dialyzed exhaustively against distilled H_2O at 4°C and freeze-dried. The lyophilized protein was suspended in 0.2 M Tris hydrochloride buffer, pH 7.7, and passed through a Sephadex G-100 gel filtration column. All protein peaks, as detected by optical density measurement, were collected separately, dialyzed against distilled H_2O, and freeze-dried. The $F(ab')_2$ fraction was identified by molecular weight on sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis.

The Fab fragment of MAb Guy's 13 was prepared by papain digestion of the IgG preparation. The lyophilized IgG was suspended at 20 mg/ml in 0.1 M sodium phosphate buffer, pH 7.0, containing 10 mM cysteine and 2 mM EDTA. Papain was added (1 mg of papain to 100 mg of IgG), and digestion was allowed to proceed at 37°C for 5 h. The reaction was stopped by increasing the pH to 8.0 by adding Tris base, and the Fab fraction was separated by affinity chromatography by using protein A-Sepharose beads. The Fab fraction was eluted in 0.1 M Tris hydrochloride buffer, pH 8.0, dialyzed against distilled H_2O, and freeze-dried.

For clinical application, the IgG fractions of MAbs were reconstituted at a concentration of 10 mg/ml in phosphate-buffered saline. The $F(ab')_2$ and Fab fractions were reconstituted at a concentration of 6.7 mg/ml in phosphate-buffered saline. All solutions were ultracentrifuged at 120,000 × *g* for 30 min and passed through a filter (pore size, 0.22 μm) before being stored at −20°C, ready for use.

The molecular weights and purity of all of the MAb preparations were ascertained by analysis on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and their activities were determined by a standard radioimmunoassay. Microtiter plates coated with SA I/II were incubated in duplicate with the various MAb preparations. Binding of the MAb was detected by using an ^{125}I-labeled, affinity-purified goat anti-mouse IgG Fab antibody (Sigma). This labeled antiserum also allows comparison between the bindings of the IgG, $F(ab')_2$, and Fab solutions. A fluid phase assay in which whole streptococcal cells were used was also performed. To confirm the presence or absence of the Fc part of the molecule, the solid-phase assay was repeated, except bound MAb was detected by using an ^{125}I-labeled, affinity-purified goat anti-mouse IgG Fc antibody (Sigma).

Detection of MAb on the tooth surface. We have attempted to determine the length of time MAb can be detected on the tooth surface following a single application of MAb. Two subjects were selected because they did not harbor detectable levels of *S. mutans* in either plaque or saliva. They had MAb Guy's 13 applied once to the buccal surfaces of all the teeth 1 h after dental prophylaxis with pumice and water. Two other subjects were sham immunized with bovine serum albumin (10 mg/ml). They were asked to avoid brushing their teeth for the next 6 days. Before and at intervals of 1, 24, 53, 74, and 148 h after the MAb application, the buccal surface of one tooth from each quadrant was gently cleaned with a sterile dental prophylaxis brush and pumice, moistened with sterile saline. The pumice and saline washings were collected and stored at −70°C until all the samples had been collected. These were then centrifuged at 8,800 × g for 2 min, and the supernatants were removed and used in a radioimmunoassay to detect mouse IgG anti-SA I/II activity. In this assay, microtiter plates coated with SA I/II (1 μg/ml) were incubated with the undiluted supernatants and MAb was detected by using an affinity-purified goat anti-mouse IgG antibody (Nordic Immunological Laboratories, Maidenhead, England) labeled with ^{125}I.

Prevention of recolonization by *S. mutans*. For these experiments, only those subjects in whom preexperimental culture of dental plaque and saliva consistently grew *S. mutans* were selected. The initial proportion of *S. mutans* was determined (see below), and chlorhexidine was then applied to the teeth for 9 consecutive days in order to eliminate *S. mutans* and other plaque organisms. The subjects were instructed to rinse their mouths with 10 ml of 0.2% (wt/vol) chlorhexidine gluconate mouthwash (Corsodyl; ICI, Macclesfield, England) for 1 min in the morning and at night. They also had 1% (wt/wt) chlorhexidine gel applied daily in stock gel application trays (Nupro; Johnson and Johnson) for 5 min. They were allowed to spit out but not to rinse the mouth for 30 min after each application. Culture of plaque and saliva at the end of the chlorhexidine treatment failed to grow detectable *S. mutans*.

A matched group of 15 subjects were then immunized as follows: 3 subjects had MAb Guy's 13 $F(ab')_2$ fragment and 3 subjects had MAb Guy's 13 Fab fragment, 2 subjects had MAb Guy's 13 IgG, 3 subjects had the control MAb Guy's 11 IgG applied, and 4 subjects had saline applied to their teeth.

One hour before the first application of MAb, the teeth of all subjects were scaled and polished. MAb or control solution was then applied directly to the teeth. A 5-μl portion was applied to the buccal, palatal, and fissural surface of each tooth by means of an Eppendorf pipette. A total of 375 μl of the immunizing solution was used in each mouth per immunization. Custom-made silicone impression trays (Optosil and Xantopren; Bayer Dental, Newbury, England) were then inserted for 5 min to prevent washing away and dilution by saliva. Application of MAb was started the day after chlorhexidine treatment was completed and was performed six times, on days 0, 3, 7, 10, 14, and 21.

Bacteriological sampling. Dental plaque was sampled with sterile probes from the smooth surfaces and interproximal areas of all first incisors and first molars as well as the natural fissures of first or second molars. The samples were transferred into transport medium (1). Unstimulated whole saliva was collected into cooled sterile bottles over a period of 4 min. All samples were kept on ice and processed within 2 h of collection.

TABLE 1. Age, sex, and dental profiles of the human volunteers[a]

Group	Age (years)	Sex (M/F)	DMFS[b]	Plaque index	Gingival index
MAb Guy's 13					
IgG	21.9 (21.5–22.3)	1/1	15.0 (0–30)	0.8 (0.5–1.0)	0.8 (0.5–1.0)
$F(ab')_2$	20.9 (20.3–21.5)	1/2	6.7 (1–16)	0.6 (0.2–0.8)	0.7 (0.3–0.9)
Fab	22.0 (21.0–23.0)	2/1	21.7 (5–32)	0.5 (0.2–1.0)	0.6 (0.3–1.0)
MAb Guy's 11 IgG	21.0 (21.0–21.0)	1/2	10.7 (6–16)	0.4 (0.1–1.0)	0.3 (0.2–0.4)
Saline	21.0 (20.8–21.3)	2/2	6.0 (0–12)	0.7 (0.4–1.0)	0.6 (0.0–1.0)

[a] All values given as means (ranges in parentheses).
[b] Decayed, missing, and filled surfaces.

Four preexperimental samples were taken over 2 weeks to determine the base-line levels of *S. mutans* before chlorhexidine was applied. Samples were taken for culture 1 day after chlorhexidine treatment was completed and then at the given intervals (see Fig. 2) up to 100 days. They were plated onto TYC agar (Lab M, Bury, United Kingdom) for enumeration of *Streptococcus sanguis* and TYC supplemented with 20% sucrose and bacitracin (0.1 mg/ml) for *S. mutans* (32). Horse blood agar supplemented with vancomycin (7.5 μg/ml) was used for veillonella species, and horse blood agar was used for the total anaerobic count. The TYC-based plates were incubated anaerobically at 37°C for 3 days, and the horse blood plates were incubated anaerobically at 37°C for 5 days. The levels of *S. mutans*, *S. sanguis*, and veillonella species were calculated as a percentage of the total anaerobic count on horse blood agar. In the case of recolonizing *S. mutans*, the proportion was then expressed as a percentage of the mean preexperimental *S. mutans* level. Identification of these organisms was made initially on the basis of colonial morphology and confirmed by the ability to produce dextran; the fermentation profiles of mannitol, sorbitol, raffinose, and melibiose; and hydrolysis of L-arginine and Voges-Proskauer test for *S. mutans* and *S. sanguis* (4, 27). Veillonellae were identified by Gram staining and inability to grow aerobically.

In vitro effects of MAb on *S. mutans* growth. The potential antibacterial action of MAb Guy's 13 was investigated in two ways. Firstly, sterile filter paper disks were impregnated with either MAb Guy's 13 or saline in various concentrations and laid onto triplicate TYC agar plates which had been inoculated with a lawn of *S. mutans* (strain K2). The plates were incubated at 37°C for 3 days. Secondly, 100 μl of each solution—Guy's 13 IgG (10 mg/ml), Guy's 13 $F(ab')_2$ (6.7 mg/ml), Guy's 13 Fab (6.7 mg/ml), Guy's 11 IgG (10 mg/ml), and normal saline—was added to 10 ml of sterile Todd Hewitt broth. This was then inoculated with 100 μl of an 18-h growth of *S. mutans* in Todd Hewitt broth and left to stand at 37°C for 18 h. The resultant growth was Gram stained and examined under oil immersion microscopy.

RESULTS

Clinical indices. The clinical indices of the volunteers in each group are summarized in Table 1. They were all dental students, and they were closely matched for age and plaque and gingival indices. A range of previous caries experience, as measured by decayed, missing, and filled surfaces, was present, but none of the volunteers had detectable active caries. There was no relationship between decayed, missing, and filled surface score and whether or not recolonization by *S. mutans* occurred.

MAbs. The activity of intact IgG and the $F(ab')_2$ and Fab digests of MAb Guy's 13 is shown in Table 2. In the solid-phase radioimmunoassay, in which purified protein antigen SA I/II was bound to the microtiter plates, MAb Guy's 13 IgG, $F(ab')_2$, and Fab preparations bound equally well to both SA I/II from *S. mutans* as well as the equivalent antigen from *S. sobrinus* when detected by a labeled goat anti-mouse IgG Fab antibody. Similarly, in the fluid-phase assay, the three MAb preparations bound equally well to the whole streptococcal cells. This confirms that the digestion and purification procedures had no effect on binding of the MAb preparations to both purified and native cell surface antigens. The control MAb, Guy's 11, also bound strongly to *S. mutans* and *S. sobrinus* antigens as well as to whole cells. The absence of intact MAb IgG in the $F(ab')_2$ and Fab preparations was confirmed in a similar radioimmunoassay in which ^{125}I-labeled goat anti-mouse IgG Fc antibody failed to detect bound MAb in these preparations at 6.7 μg/ml (Table 2) or at a concentration 10-fold higher (data not shown), although the intact MAb IgG gave high binding.

Detection of MAb on the tooth surface. Anti-SA I/II antibodies were not detected with the ^{125}I-labeled goat anti-mouse antibody in the samples collected from the teeth of control subjects sham immunized with bovine serum albumin or in either subject prior to the application of MAb Guy's 13 (Table 3). One hour after application of MAb, there was a high level of SA I/II-specific mouse IgG detectable in

TABLE 2. Radioimmunoassay to compare the activities of the MAb preparations

Antigen	% Binding[a] to:				
	Guy's 13 IgG (10 μg/ml)	Guy's 13 $F(ab')_2$ (6.7 μg/ml)	Guy's 13 Fab (6.7 μg/ml)	Guy's 11 IgG (10 μg/ml)	Normal mouse serum (10^{-3} dilution)
SA I/II (*S. mutans*)[b]	11.5	11.7	11.6	10.9	0.2
SA I/II (*S. sobrinus*)[b]	10.8	11.6	11.6	11.0	0.1
S. mutans cells[b]	13.1	12.2	11.6	9.2	0.3
S. sobrinus cells[b]	18.8	15.3	17.8	14.6	0.6
SA I/II (*S. mutans*)[c]	8.4	0.2	0.2	7.6	0.1

[a] Results are expressed as mean percentage of binding of labeled antisera to MAb preparations incubated with either purified antigen or whole streptococcal cells.
[b] The bound MAb was detected with ^{125}I-labeled, affinity-purified goat anti-mouse IgG (Fab) antibodies.
[c] The bound MAb was detected with ^{125}I-labeled, affinity-purified goat anti-mouse IgG (Fc) antibodies.

TABLE 3. Detection of MAb on teeth by radioimmunoassay following a single application of MAb[a]

Time after application (hours)	% Binding of ^{125}I-labeled antiserum to washings incubated with SA I/II	
	Immunized (MAb, Guy's 13)	Control (bovine serum albumin)
Preapplication	0.01	0.02
1	10.74	0.08
24	5.42	0.07
53	1.93	0.08
74	0.25	0.05
148	0.03	0.04

[a] Mean of duplicate assays for two subjects.

the washings from the teeth of the immunized subjects. This decreased gradually in subsequent samples, but significant levels were still detectable 3 days after the original application. The mouse IgG was not detectable 6 days after the MAb was applied.

Prevention of recolonization of *S. mutans*. All the subjects chosen for this investigation originally harbored consistently detectable levels of *S. mutans* in plaque and saliva, as measured over the 2 weeks before the experiment was started. The subjects were evenly allocated into experimental and control groups according to age and sex. The chlorhexidine treatment over 9 days was effective in reducing the *S. mutans* levels in plaque and saliva to undetectable levels in all subjects (Fig. 1). In those subjects sham immunized with either saline or MAb Guy's 11, *S. mutans* began to reappear in both plaque and saliva almost immediately and was readily detectable by culture by the end of the second week after the chlorhexidine was discontinued (days 9 to 15). The level of *S. mutans* expressed as a percentage of the original proportion of *S. mutans* continued to rise and reached 100% or more by the end of 100 days of the experimental period. In contrast, subjects who had been immunized with either MAb Guy's 13 intact IgG or the $F(ab')_2$ fragment did not recolonize with *S. mutans* even after 100 days in plaque or saliva. However, in the group that had been immunized with the Guy's 13 Fab fragment, no protection was seen and recolonization by *S. mutans* occurred at a similar rate to that of the control subjects. It appears that while intact IgG and the $F(ab')_2$ fragment can prevent colonization of *S. mutans*, the Fab fragment loses this function.

Unlike the effect of chlorhexidine in eliminating detectable

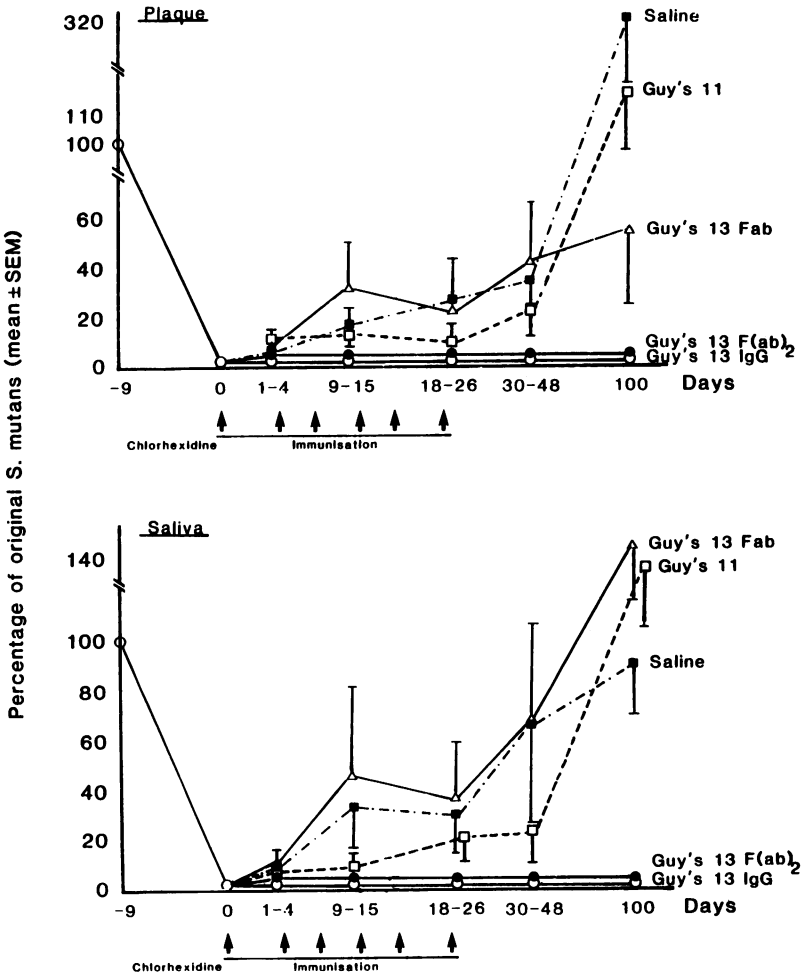

FIG. 1. The proportion of recolonizing indigenous *S. mutans* in plaque and saliva. The *S. mutans* proportions are expressed as mean ± standard error of the mean percentage of the *S. mutans* preexperimental level. Preexperiment levels (day 9) are the mean proportions of 4 samples taken over 14 days.

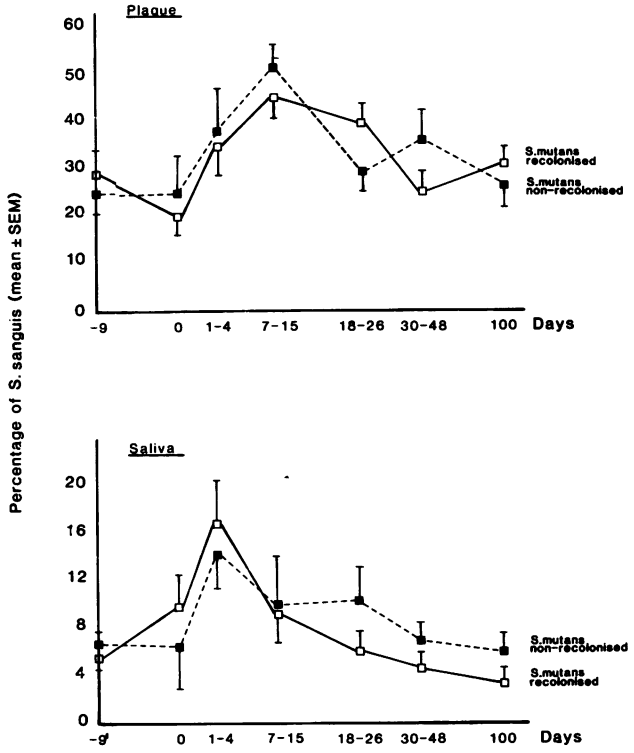

FIG. 2. The proportions of *S. sanguis* are presented for (i) a *S. mutans* recolonized group which received saline, MAb Guy's 11, or Guy's 13 Fab fragment (□) and (ii) a *S. mutans* nonrecolonized group which received MAb Guy's 13 IgG or F(ab')$_2$ fragment (■). The results are expressed as percentage of *S. sanguis* of the total anaerobic count. Preexperiment levels (day 9) are the mean proportions of 4 samples taken over 14 days.

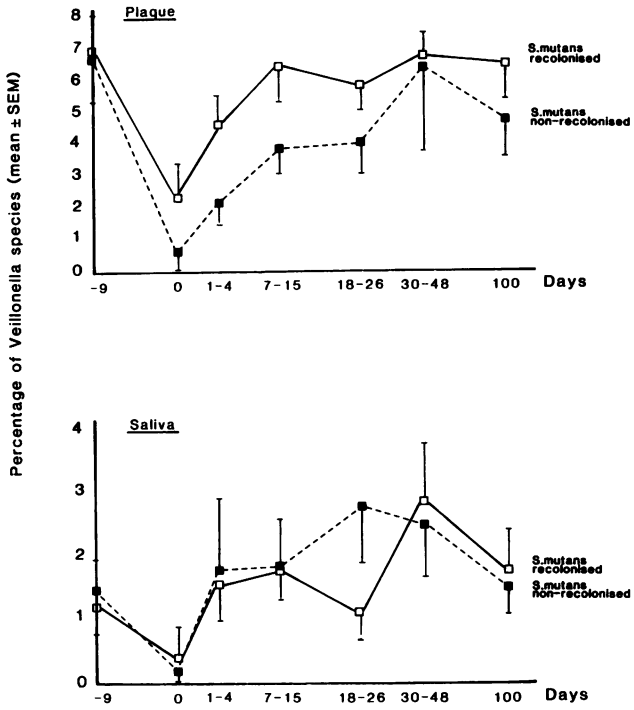

FIG. 3. The proportion of veillonella species are presented for (i) a *S. mutans* recolonized group which received saline, MAb Guy's 11, or Guy's 13 Fab fragment (□) and (ii) a *S. mutans* nonrecolonized group which received MAb Guy's 13 IgG or F(ab')$_2$ fragment (■). The results are expressed as percentage of veillonellae of the total anaerobic count. Preexperiment levels (day 9) are the mean proportions of 4 samples taken over 14 days.

S. mutans, *S. sanguis* was not completely cleared from plaque or saliva (Fig. 2). Following application of MAb Guy's 13 or control solutions, there was a transient increase in the proportion of *S. sanguis* in both groups up to days 7 to 15 for plaque and days 1 to 4 in saliva. However, the proportion of *S. sanguis* subsequently returned to the preexperimental levels. There was no significant difference in the proportion of *S. sanguis* before and after immunization or between the MAb-immunized subjects who were not recolonized by *S. mutans* and the controls who were not protected and were recolonized by *S. mutans* (Fig. 2). This is in marked contrast with the very significant difference observed with *S. mutans* (Fig. 1). The results are consistent with the MAb action being specific against *S. mutans* and having little effect on *S. sanguis*.

Chlorhexidine decreased or eliminated detectable veillonella species in dental plaque and saliva, as was found with *S. mutans* (Fig. 3). Although after the chlorhexidine was discontinued, the proportion of veillonellae increased in the plaque samples, there was a trend in the subjects who were not recolonized by *S. mutans* for the subsequent levels to remain lower, even after 100 days, than the preexperimental values. The difference between pre- and postexperimental levels did not, however, reach statistical significance. In the subjects whose teeth were recolonized with *S. mutans*, the proportion of veillonellae at 100 days was the same as the preexperimental levels. In the saliva of the subjects whose teeth were not recolonized, there appeared to be a transient rise in the proportions of veillonellae up to about 18 to 26 days, which then fell back to the preexperimental values (Fig. 3). As with *S. sanguis*, there was no significant difference between the two groups, unlike that found with *S. mutans*.

In vitro effects of MAbs on *S. mutans* growth. None of the MAb preparations prevented *S. mutans* growth on agar plates. A dense, even lawn of bacterial growth was seen on all plates, whether or not MAb-impregnated filter disks were present. The growth of *S. mutans* in liquid culture was however markedly affected by the presence of MAb Guy's 13. Whereas *S. mutans* grown in the presence of saline resulted in a uniform, dense culture broth, the bacterial cells grown in the presence of MAb Guy's 13 were clearly aggregated and clumped together at the bottom of the growth flask. Microscopically the difference was striking; *S. mutans* grown with saline but without MAb formed short chains (Fig. 4A), but when intact MAb Guy's 13 was present, much longer chains were formed, which became entangled into bacterial clusters (Fig. 4B). Indeed, MAb Guy's 13 F(ab')$_2$ fragments also caused long chaining and aggregation, whereas cells grown with the monovalent Fab did not appear different from those grown with saline. When the two different growths were vortexed, there was no difference in optical density and an equal number of viable cells were found in both types of culture. MAb Guy's 11 had no effect on *S. mutans* growth, and long chains were not formed.

DISCUSSION

In previous studies we have shown that MAb specific for *S. mutans* can prevent colonization of exogenous *S. mutans*

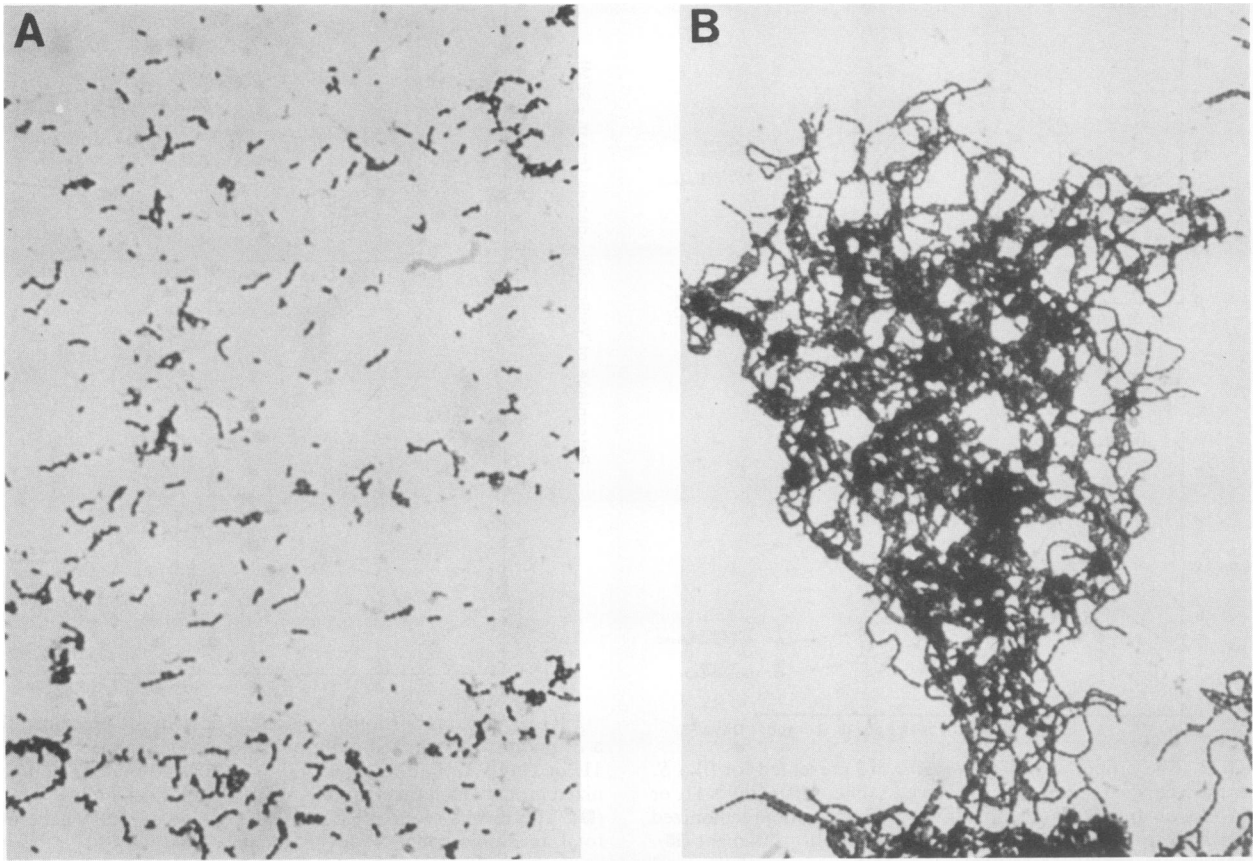

FIG. 4. Gram-stained *S. mutans* cells viewed under oil immersion light microscopy. The *S. mutans* were grown for 18 h in Todd-Hewitt broth with control MAb (A) or with MAb Guy's 13 (B).

in human subjects (18). Recolonization by indigenous *S. mutans* can also be prevented by MAb if the oral flora is first depleted by using chlorhexidine (17). In this series of experiments, the protective function of *S. mutans*-specific MAb has been demonstrated not to require the entire IgG molecule. The $F(ab')_2$ fragment of MAb Guy's 13 was as effective as the intact IgG in preventing recolonization of *S. mutans*. $F(ab')_2$ differs from IgG in that the Fc portion, which carries the CH_2 and CH_3 domains, has been removed. The function of these domains is in binding to phagocytes as well as activating complement (15). This suggests that the mode of action of the MAb in preventing streptococcal colonization might be independent of these two biological properties. The finding that complement activation is not essential is consistent with our previous observation that MAbs of both IgG2a and IgG1 subclasses were protective (17).

The Fab fragment of IgG, however, failed to prevent colonization of *S. mutans*. This may be due to the Fab being a monovalent molecule, unlike the bivalent $F(ab')_2$ and IgG molecules. Alternatively, the smaller antibody fragment may be more susceptible to proteolysis than the larger $F(ab')_2$ or IgG molecule. In the in vitro studies of MAb and *S. mutans* growth, the intact MAb had no bacteriostatic or bactericidal activity but caused long chaining of the streptococcal cells, which resulted in aggregation of the cells. It is significant that both intact IgG and the $F(ab')_2$ fragments induced long chaining but the Fab fragments did not.

Long chaining has been reported in the past (31) with Lancefield group A streptococci grown in the presence of specific antiserum. Long chains have also been observed in *S. mutans* mutants (23), in which the long chains also resulted in aggregation of the bacterial cells. There was no effect on growth rate, however, nor was there any consistent difference in the ability of the mutants to adhere in vivo. Although the mechanism of the long chain phenomenon is not known, Hahn and Cole (7) suggested the most likely cause was the promotion of the "end-to-end" agglutination by an antibody specific for M protein on the surface of *Streptococcus pyogenes*; indeed, Fab molecules failed to induce long chaining. A further mechanism for long chaining, which involves the elimination of a dechaining factor, has been suggested. Lysozyme has been shown to dechain *S. mutans* without detectable growth inhibition, and this was thought to be due to muraminidase activity (9). We have now demonstrated long chaining occurring in *S. mutans* incubated with specific MAb to the SA I/II. This is an important protein which is densely distributed on the cell surface (22, 33), appears to be a virulence factor (5), and may function in adherence to teeth (10, 11, 19). Since the bivalent nature of $F(ab')_2$ or intact IgG is essential for long chaining, unlike the monomeric Fab fragments, long chaining might be accounted for by binding of adjacent streptococcal cells by the two arms of the antibody molecule of IgG or $F(ab')_2$. Long chaining did not, however, inhibit growth or division of *S. mutans* in vitro, but under in vivo conditions, long chaining and aggregation of *S. mutans* might be unfavorable to growth and the aggregated cells might be more readily removed by self-cleansing mechanisms or by phagocytosis. Clearly, further work is required to establish the mechanism of chaining elicited by the MAb to SA I/II.

A surprising feature of prevention of recolonization of *S. mutans* by MAb has been the long duration of protection. None of the subjects from previous experiments (17) have recolonized over a period of 1 year, and 2 subjects have now reached 2 years postimmunization and are still free of *S. mutans*. Of the present five subjects, although the results are given only up to day 100, all have remained clear of *S. mutans* for periods in excess of 1 year. This is despite having been immunized only on six occasions over the first 3 weeks.

We have also shown that MAb can persist for up to 3 days on the tooth surface and is able to bind SA I/II but that after 3 days, the MAb is no longer detectable by a radioimmunoassay. We have previously proposed that the long-term protection may be due to a shift in the oral flora and development of colonization resistance towards *S. mutans* (17). Chlorhexidine is a broad-spectrum antibacterial agent which has the overall effect of reducing the total number of bacteria in the oral cavity. Once the chlorhexidine is stopped, recolonization by the plaque organisms takes place, but in the presence of MAb to *S. mutans*, the latter is specifically prevented, allowing other organisms to occupy the vacated ecological niche. Once the new balance of the microflora is established, there is no available niche for *S. mutans* and a colonization resistance develops.

We have now investigated the changes in the proportions of *S. sanguis* and veillonella species during the recolonization experiments. These show that after a transient disturbance in the proportions of *S. sanguis* and veillonellae, there is a gradual return close to the preexperimental values both in plaque and saliva. The initial rise in the proportions of *S. sanguis* in both groups after chlorhexidine was stopped is consistent with *S. sanguis* being an early colonizer of teeth (3). However, the subsequent return of *S. sanguis* to preexperimental values is not consistent with *S. sanguis* being one of the organisms filling the ecological niche vacated by *S. mutans*, unless the quantitative shift from *S. mutans* to *S. sanguis* is too small to be detectable by the method used.

MAb had no consistent effect on the proportion of veillonella species, although in the plaque of the group of subjects who failed to recolonize *S. mutans*, there was a trend for the final proportions of veillonellae to be lower than the preexperimental values. On the basis of the known ability of veillonellae to thrive in the presence of acid produced by *S. mutans* (2), a decrease of *S. mutans* in plaque might also result in a parallel decrease in veillonella species. The striking effect of MAb in preventing colonization of *S. mutans* and the lack of a similar effect on *S. sanguis* or veillonellae is consistent with the specific effect of the MAb on colonization of *S. mutans*.

Local passive immunization is potentially safer than other routes of immunization, as it bypasses the need for active immunization and any undesirable systemic immune response. Unlike other examples of passive immunization, such as gamma globulin for infectious hepatitis, the protective effects appear to last long after the antibody has been cleared. A short course of treatment with the MAb appears to confer long-term protection, possibly due to a shift in the oral microflora, which may result in the development of specific colonization resistance.

ACKNOWLEDGMENT

We acknowledge support by the Newland Pedley Fund.

LITERATURE CITED

1. **Bowden, G. H., and J. M. Hardie.** 1971. Anaerobic organisms from the human mouth, p. 177–199. *In* D. A. Shapton and R. G. Board (ed.), Isolation of anaerobes. Academic Press, Inc. (London) Ltd., London.
2. **Bowden, G. H., J. M. Hardie, A. A. S. McKee, P. P. Marsh, E. D. Fillery, and G. L. Slack.** 1976. The microflora associated with developing carious lesions of the distal surfaces on the upper premolars in 13–14 year old children, p. 223–242. *In* H. M. Stiles, W. J. Loesche, T. C. O'Brien (ed.), Microbiological aspects of dental caries. Information Retrieval Inc., Washington, D.C.
3. **Carlsson, J., H. Grahnen, G. Jonsson, and S. Wikner.** 1970. Establishment of *Streptococcus sanguis* in the mouths of infants. Arch. Oral Biol. **15**:1143–1148.
4. **Cowan, S. T., and K. J. Steel.** 1974. *In* S. T. Cowan (ed.), Manual for the identification of medical bacteria, 2nd ed. Cambridge University Press, Cambridge.
5. **Curtiss, R., III.** 1985. Genetic analysis of *Streptococcus mutans* virulence. Curr. Top. Microbiol. Immunol. **118**:253–277.
6. **Emilson, C.-G., and B. Krasse.** 1985. Support for and implications of the specific plaque hypothesis. Scand. J. Dent. Res. **93**:96–104.
7. **Hahn, J. J., and R. M. Cole.** 1962. Studies on the mechanism of the long chain phenomenon of group A streptococci. J. Exp. Med. **117**:583–594.
8. **Hamada, S., and H. D. Slade.** 1983. Biology, immunology, and cariogenicity of *Streptococcus mutans*. Microbiol. Rev. **44**:331–384.
9. **Iacono, V. J., T. P. Byrnes, I. T. Crawford, B. L. Grossbard, J. J. Pollock, and B. J. Mackay.** 1985. Lysozyme-mediated de-chaining of *Streptococcus mutans* and its antibacterial significance in an acidic environment. J. Dent. Res. **64**:48–53.
10. **Koga, T., N. Okahashi, I. Takahashi, T. Kanamoto, H. Asakawa, and M. Iwaki.** 1990. Surface hydrophobicity, adherence, and aggregation of cell surface protein antigen mutants of *Streptococcus mutans* serotype c. Infect. Immun. **58**:289–296.
11. **Lee, S. F., A. Progulske-Fox, G. W. Erdos, D. A. Piacentini, G. Y. Ayakawa, P. J. Crowley, and A. S. Bleiweis.** 1989. Construction and characterization of isogenic mutants of *Streptococcus mutans* deficient in major surface protein antigen P1 (I/II). Infect. Immun. **57**:3306–3313.
12. **Lehner, T., J. Ma, K. Grant, and R. Smith.** 1986. Passive dental immunization by monoclonal antibodies against Streptococcus mutans antigen in the prevention of dental caries, p. 347–356. *In* T. Lehner and G. Cimasoni (ed.), Borderland between caries and periodontal disease. Editions Médecine et Hygiène, Geneva.
13. **Lehner, T., J. Caldwell, and R. Smith.** 1985. Local passive immunization by monoclonal antibodies against streptococcal antigen I/II in the prevention of dental caries. Infect. Immun. **50**:796–799.
14. **Lehner, T., M. W. Russell, and J. Caldwell.** 1980. Immunisation with a purified protein from *Streptococcus mutans* against dental caries in rhesus monkeys. Lancet **i**:995–996.
15. **Leslie, R. G. Q., and S. Cohen.** 1973. The active sites of immunoglobulin molecules. *In* Essays in fundamental immunology I. Blackwell Scientific Publications, Ltd., Oxford.
16. **Loe, H., and J. Silness.** 1963. Periodontal disease in pregnancy. I. Prevalence and severity. Acta Odontol. Scand. **21**:532–551.
17. **Ma, J. K.-C., M. Hunjan, R. Smith, and T. Lehner.** 1989. Specificity of monoclonal antibodies in local passive immunization against *Streptococcus mutans*. Clin. Exp. Immunol. **77**: 331–337.
18. **Ma, J. K.-C., R. Smith, and T. Lehner.** 1987. Use of monoclonal antibodies in local passive immunization to prevent colonization of human teeth by *Streptococcus mutans*. Infect. Immun. **55**:1274–1278.
19. **McBride, B. C., M. Song, B. Krasse, and J. Olsson.** 1984. Biochemical and immunological differences between hydrophobic and hydrophilic strains of *Streptococcus mutans*. Infect. Immun. **44**:68–75.
20. **Michalek, S. M., R. L. Gregory, C. C. Harman, J. Katz, G. J. Richardson, T. Hilton, S. T. Filler, and J. R. McGhee.** 1987. Protection of guotobiotic rats against dental caries by passive immunization with milk antibodies to *Streptococcus mutans*. Infect. Immun. **55**:2341–2347.

21. **Mitchell, C. G., R. Smith, and T. Lehner.** 1987. Recognition of carbohydrate and protein epitopes by monoclonal antibodies to a cell wall antigen from *Streptococcus mutans*. Infect. Immun. **55**:810–815.
22. **Moro, I., and M. W. Russell.** 1983. Ultrastructural localization of protein antigens I/II and III in *Streptococcus mutans*. Infect. Immun. **41**:410–413.
23. **Murchison, H., S. Larrimore, S. Hull, and R. Curtiss III.** 1982. Isolation and characterization of *Streptococcus mutans* mutants with altered cellular morphology or chain length. Infect. Immun. **38**:282–291.
24. **Russell, R. R. B.** 1979. Wall associated protein antigens of *Streptococcus mutans*. J. Gen. Microbiol. **114**:109–115.
25. **Russell, M. W., L. A. Bergmeier, E. D. Zanders, and T. Lehner.** 1980. Protein antigens of *Streptococcus mutans*: purification and properties of a double antigen and its protease-resistant component. Infect. Immun. **28**:486–493.
26. **Russell, M. W., and T. Lehner.** 1978. Characterization of antigens extracted from cells and culture fluids of *Streptococcus mutans* serotype c. Arch. Oral Biol. **23**:7–15.
27. **Shklair, I. L., and H. J. Keene.** 1974. A biochemical scheme for the separation of the five varieties of *Streptococcus mutans*. Arch. Oral Biol. **19**:1079–1081.
28. **Silness, J., and H. Loe.** 1964. Periodontal disease in pregnancy. II. Correlation between oral hygiene and periodontal condition. Acta Odontol. Scand. **22**:121–135.
29. **Smith, R., and T. Lehner.** 1981. A radioimmunoassay for serum and gingival fluid antibodies to a purified antigen from *Streptococcus mutans*. Clin. Exp. Immunol. **43**:417–428.
30. **Smith, R., and T. Lehner.** 1989. Characterization of monoclonal antibodies binding to common protein epitopes on the cell surface of *Streptococcus mutans* and *Streptococcus sobrinus*. Oral Microbiol. Immunol. **4**:153–158.
31. **Stollerman, G. H., and R. Ekstedt.** 1957. Long chain formation by strains of group A streptococci in the presence of homologous antiserum: a type specific reaction. J. Exp. Med. **106**:345–355.
32. **Van Palenstein Helderman, W. H., M. Ijsseldijk, and J. H. J. Huis in't Veld.** 1983. A selective medium for the two major subgroups of the bacterium *S. mutans* isolated from human dental plaque and saliva. Arch. Oral Biol. **28**:599–603.
33. **Zanders, E. D., and T. Lehner.** 1981. Separation and characterization of a protein antigen from cells of *Streptococcus mutans*. J. Gen. Microbiol. **122**:217–225.