A SEQUENTIAL BACTERIOLOGICAL AND SEROLOGICAL INVESTIGATION OF RHESUS MONKEYS IMMUNISED AGAINST DENTAL CARIES WITH *STREPTOCOCCUS MUTANS*

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MICROBIOLOGICAL investigations in man strongly support an association of *Streptococcus mutans* with dental caries (de Stoppelaar, van Houte and Dirks, 1969; Littleton, Kakehashi and Fitzgerald, 1970; Shklair, Keene and Simonson, 1972; Loesche *et al.*, 1975). Few sequential studies have been performed, but Krasse *et al.* (1968) found that children with the highest number of *S. mutans* in plaque were those who subsequently developed caries, and Ikeda, Sandham and Bradley (1973) reported that the development of caries in children was preceded by a rise in the number of *S. mutans*.

The rhesus monkey is a useful model for investigating dental caries (Lehner, Challacombe and Caldwell, 1975a) because the development of the disease is comparable with that found in man, in that there is an increase in the number of naturally acquired *S. mutans* if the animals are maintained on a "human" type of diet. Immunisation with *S. mutans* leads to a reduction in caries and in the number of *S. mutans* (Lehner, Challacombe and Caldwell, 1975c). Bowen *et al.* (1975) also found reduced numbers of *S. mutans* in the plaque of rhesus monkeys immunised against caries. In rats, significant reduction in caries after immunisation was accompanied by smaller numbers of recoverable *S. mutans* (Hayashi, Shklair and Bahn, 1972). Taubman and Smith (1974) found significantly less *S. mutans* in only one of seven groups of immunised animals, and Tanzer, Hageage and Larson (1973) found no reduction after immunisation. Fluctuations in the number of *S. mutans* might account for this apparent variability.

The aim of this investigation was to perform sequential enumerations of *S. mutans* throughout the experimental period, and so to determine whether differences between the number of *S. mutans* in samples from immunised and control rhesus monkeys were maintained. We counted the number of *S. mutans* and *S. sanguis* in samples from the teeth, and assayed antibodies against *S. mutans* in the serum and saliva of immunised and sham-immunised animals.

MATERIALS AND METHODS

Preparation of vaccine

The vaccine was prepared from a strain of *S. mutans* (serotype c) isolated from a monkey that had acquired it and had developed caries whilst on a "human" type of carbohydrate diet. A 24-h Todd-Hewitt broth culture was harvested by centrifugation at 6000 g. The

Received 26 Aug. 1976; accepted 30 Oct. 1976.

cells were washed three times with sterile saline (NaCl 0.85% w/v), suspended in a 0.6% v/v dilution of 40% formalin in 0.1M phosphate-buffered saline, pH 7.3, overnight at room temperature. After washing three times with saline the suspension was adjusted to a concentration of 2×10^9 organisms per ml and stored in a 0.2% v/v dilution of 40% formalin in the same buffer. For immunisation, 1×10^9 organisms (in 0.5 ml) was mixed with 0.5 ml of Freund's incomplete adjuvant.

Animals, diet and immunisation schedule

A series of six rhesus monkeys was divided into two groups, with two males and one female in each. All the animals had a fully erupted deciduous dentition and one animal in each group also had the first permanent molars. The monkeys were given a "human" type of diet, as described earlier (Lehner *et al.*, 1975a), from 2 days after the beginning of immunisation until the end of the experiment. Three animals were given subcutaneous injections of 0.5 ml of the vaccine into the left arm and 0.5 ml into the right thigh. The controls were sham-immunised at the same sites with PBS. Thirty weeks later, similar injections were given into the right arm and left leg of immunised and control monkeys. The animals were examined at 3- and later at 6-weekly intervals up to the 45th week. Samples of blood and whole saliva were collected as described previously (Lehner, Challacombe and Caldwell, 1975b), and the number of carious cavities (the "caries score") was determined.

Bacteriological examination

Collection of samples. Four samples were collected from each monkey at each examination; smooth-surface plaque, fissure plaque, crevicular fluid and saliva. Smooth-surface plaque was removed with a sterile probe from the cervical and approximal sites of the upper left first and second deciduous molars. Crevicular fluid was collected from the same site as previously described (Lehner *et al.*, 1975c). The fissures of the same teeth were sampled and whole saliva was collected into sterile petri dishes. Each of these samples was immediately transferred to 5 ml of transport medium (Bowden and Hardie, 1971), kept at 4°C and cultured within 24 h.

Cultural methods. The samples were dispersed by vigorous shaking with sterile glass beads (3-mm diameter) on a vortex mixer (Fisons Ltd, Loughborough, Leicestershire) for 60 s. Three 10-fold dilutions were made in transport medium, and 0.1 ml of each dilution was inoculated in duplicate on to plates of TYC medium (de Stoppelaar, van Houte and de Moor, 1967). The plates were incubated for 4 days at 37°C in an atmosphere of $H_2 + 5\% v/v CO_2$.

TABLE I

Proportion of Streptococcus mutans in samples collected 1½ years after immunisation or sham-immunisation

Sample	Percentage ($\pm$ S.E.) of <i>S. mutans</i> in the total colony count on TYC medium in		<i>t</i>	P
	immunised animals	control animals		
Cervical plaque	18 ($\pm$ 9.3)	62 ($\pm$ 15.0)	2.499	NS
Crevicular fluid	30 ($\pm$ 21.0)	61 ($\pm$ 16.0)	1.180	NS
Fissures	23 ($\pm$ 10.7)	46 ($\pm$ 11.9)	1.419	NS
Saliva	8 ($\pm$ 4.6)	47 ($\pm$ 4.3)	6.130	<0.005

t = Value obtained by Student's *t* test.
P = Significance.
NS = Not significant.

TABLE II
Rate of colonisation by *S. mutans* in samples from immunised and control animals

Sample	Mean weekly increase ($\pm$ S.E.) in the percentage of <i>S. mutans</i> in the total colony count in		<i>t</i>	P
	immunised animals	control animals		
Cervical plaque	0.548 ($\pm$ 0.35)	1.248 ($\pm$ 0.46)	1.204	NS
Crevicular fluid	0.510 ($\pm$ 0.22)	1.945 ($\pm$ 0.35)	3.434	<0.05
Fissures	0.831 ($\pm$ 0.63)	1.237 ($\pm$ 0.39)	0.546	NS
Saliva	0.176 ($\pm$ 0.07)	1.174 ($\pm$ 0.24)	3.953	<0.02

t = Value obtained by Student's *t* test.

P = Significance.

NS = Not significant.

Enumeration, isolation and identification of S. mutans and other dextran-producing streptococci. After incubation, suitable plates were selected and the colonies counted. Colonies that produced extracellular polysaccharide (EPS) were differentiated according to their colonial morphology and enumerated. Representative colonies were subcultured for positive identification. Strains that fermented mannitol and sorbitol were designated *S. mutans*. Strains of *S. mutans* were serotyped by the fluorescent antibody technique with antisera against serotypes a, b, c and d of *S. mutans* conjugated with fluorescein isothiocyanate. EPS-producing strains that failed to ferment mannitol and sorbitol were identified according to the methods described by Carlsson (1968). The number of colonies of EPS producing streptococci was expressed as a percentage of the total number of colonies on TYC medium.

Serological methods

An extract (termed HACS) from the culture supernate of the immunising strain of *S. mutans* was prepared by chromatography on hydroxyapatite, as described previously (Lehner *et al.*, 1975b). Serum antibodies against HACS were assayed by complement fixation (Lehner *et al.*, 1975b), passive haemagglutination (Challacombe, Guggenheim and Lehner, 1973) and immunodiffusion (Ouchterlony, 1964). Salivary haemagglutinating antibodies were determined by a microtitration method (Challacombe, Lehner and Guggenheim, 1972).

RESULTS

S. mutans and other dextran-producing streptococci

S. mutans was found in 12 of 24 samples before immunisation and institution of the high-carbohydrate diet, but it formed less than 10% of the total colony count (fig. 1). The proportion of *S. mutans* in the flora of both immunised and control animals increased over the experimental period at the four sampling sites. Apart from crevicular fluid at week 3, consistently smaller proportions of *S. mutans* were found in all samples from the immunised animals than in corresponding samples from the controls. The difference reached significant levels in crevicular fluid ($P < 0.05$) and saliva ($P < 0.01$) (fig. 1). Final samples were taken at the end of the experiment 1½ years after immunisation. The

FIG. 1.—The occurrence of *Streptococcus mutans* in control (○) and immunised (●) animals in (a) cervical plaque, (b) crevicular fluid, (c) fissures and (d) saliva, expressed as a percentage of the total colony counts.

reduction in the proportion of *S. mutans* in the immunised animals was still apparent in all the samples (table I).

The difference in the time of colonisation between immunised animals and controls was significant for cervical plaque, crevicular fluid and saliva ($P < 0.05$) but not for samples from the fissures. The mean delay was 24 weeks in cervical plaque, 17 weeks in crevicular fluid and 14 weeks in saliva (fig. 2). The rate of colonisation by *S. mutans* was calculated by plotting the proportional counts against time and determining the slope of the regression line for each animal. The rate was greater in the controls than in the immunised animals for each of the four samples (table II) and reached significant levels with crevicular fluid ($P < 0.05$) and saliva ($P < 0.02$).

EPS-producing streptococci that were not identified as *S. mutans* were found to be *S. sanguis* groups IA and IIB (Carlsson, 1968). These corresponded to *S. mitior* and *S. sanguis* of Colman and Williams (1972) and *S. sanguis* types II and I of Hardie (1975). In contrast to the findings with *S. mutans*, there was no significant difference between the proportion of *S. sanguis* in the flora of immunised and control animals throughout the experiment. However, an inter-relationship was found between counts of *S. sanguis* and *S. mutans* in both

FIG. 2.—Time of initial colonisation with *S. mutans* in control (C) and immunised (I) animals.

groups. The proportion of *S. sanguis* decreased as the proportion of *S. mutans* increased, and a “cross-over point” was found where the percentage of *S. mutans* first exceeded the percentage of *S. sanguis* (fig. 3). This point was delayed in the immunised animals in comparison with the controls for a mean of 28 weeks in cervical plaque ($P < 0.05$) (fig. 3a). With crevicular fluid, a delay was also apparent but did not reach statistical significance (fig. 3b). Samples from fissures (fig. 3c) showed more variability, particularly in the immunised animals, and no difference in the cross-over point could be detected. A cross-over point in saliva was found at 22 weeks in the control animals (fig. 3d), but the values in the immunised animals were low for both *S. mutans* and *S. sanguis*, and no cross-over point had been detected by 45 weeks.

Caries score

The smooth-surface-caries score was lower in the immunised than in the control animals (fig. 4). After 45 weeks, the mean number of carious cavities in the controls was $6.3 (\pm 1.9)$ and in the immunised animals $2.3 (\pm 1.5)$; one of the immunised monkeys was still caries-free. No fissure caries was detected by this time in any of the animals. A significantly positive correlation was found between the smooth-surface-caries score and the proportion of *S. mutans* in all the cervical-plaque samples from both immunised and control animals ($P < 0.01$)

FIG. 3.—Sequential comparison of proportions of *S. mutans* (●) and *S. sanguis* (○) in (a) cervical plaque, (b) crevicular fluid, (c) fissures and (d) saliva of control and immunised animals. Arrow indicates “cross-over point” (see text).

FIG. 4.—Sequential serum and salivary antibody titres and the caries score in immunised and control animals: (○) serum complement-fixing antibodies; (▲) serum haemagglutinating antibodies; serum precipitating antibodies in 1 (+), 2 (++) or 3 (+++) animals; (■) salivary haemagglutinating antibodies and (●) caries score. Arrows (↑) indicate time of immunisation with *S. mutans* vaccine and saline.

FIG. 5.—Relationship between the proportions of *S. mutans* and *S. sanguis* in cervical plaque samples and the caries score. Correlation for *S. mutans* is 0.367 ($P < 0.01$) and for *S. sanguis* 0.003 (not significant).

(fig. 5). In contrast, no relationship between the proportional frequency of *S. sanguis* and the caries score was apparent (fig. 5).

Antibody response

Complement fixing (CF) antibodies of mean $\log_2$ 0.8 (± 0.3) and haemagglutinating (HA) antibodies of $\log_2$ 4.7 (± 1.0) were found in the sera before immunisation; there was no significant difference between titres in the monkeys subsequently to be immunised and the control animals (fig. 4). At 3 weeks, the immunised animals had developed a mean CF titre of $\log_2$ 5.3 (± 0.3) and a mean HA titre of 13.0 (± 0) in comparison with $\log_2$ 0.3 (± 0.3) and $\log_2$ 4.0 (± 1.2) in the controls. A mean CF antibody titre of greater than $\log_2$ 5.0 was maintained in the immunised group until week 39 and had fallen to $\log_2$ 4.0 (± 1.0) at week 45. HA antibodies were maintained at $\log_2$ 10.0 or greater throughout the experimental period. In the controls, CF antibodies did not increase above $\log_2$ 2.0, but HA antibodies showed a slight increase in titre from $\log_2$ 3.7 (± 0.7) at week 6 to $\log_2$ 6.0 (± 0.6) at week 39. No precipitating antibodies were found before immunisation, but at 6 weeks one immunised monkey showed precipitating antibodies against HACS and by 9 weeks all three immunised animals gave positive results. This was maintained until week 39, after which two of the three animals still gave positive results.

In saliva, in contrast to serum, no significant difference between the two groups in the titres of haemagglutinating antibodies against HACS was found.

Three weeks after the first immunisation, both the immunised and control animals had a mean titre of $\log_2 1.3 (\pm 0.9)$; after the second injection, the mean titres were $\log_2 3.0 (\pm 0.6)$ and $\log_2 2.3 (\pm 0.7)$ respectively.

DISCUSSION

A sequential study over a period of 45 weeks showed that reduction in smooth-surface caries of rhesus monkeys immunised with *S. mutans* was associated with consistently lower proportions of *S. mutans* in plaque, fissures, saliva and crevicular-fluid washings than was found in similar samples from sham-immunised controls. This reduction in the frequency of *S. mutans* in immunised animals is consistent with previous findings (Lehner *et al.*, 1975c) in which sampling was carried out 24 weeks after immunisation.

The variability of previous reports (Hayashi *et al.*, 1972; Tanzer *et al.*, 1973; Taubman and Smith, 1974; Bowen *et al.*, 1975) may be due to fluctuation in the number of *S. mutans* in plaque samples, and the quantitative errors inherent in single estimations and the technique of bacteriological counting. In a longitudinal study, such fluctuations can be detected and a continuous assessment of differences between immunised and control animals can be made. In the present study, there was a delay in the onset of colonisation with *S. mutans* in the immunised animals and a slower rate of colonisation over the period of study in comparison with the controls. Furthermore, as the proportion of *S. mutans* increased in all animals, the proportion of *S. sanguis* decreased, and a cross-over point could be determined when the number of *S. mutans* first exceeded the number of *S. sanguis*. This cross-over point occurred later in the immunised animals than in the controls. An inverse relationship between the frequency of *S. mutans* and *S. sanguis* has also been noted in plaque from rhesus monkeys (Cornick and Bowen, 1972) and man (de Stoppelaar, van Houte and Dirks, 1970). Cornick and Bowen (1972) postulated that the fall in pH after the ingestion of sucrose favoured the more aciduric *S. mutans* or that there was a difference in their adherence properties. Bacterial antagonism may play a part in the inverse relationship between the two strains of streptococci. Bacteriocines or bacteriocine-like substances have been demonstrated in some strains of *S. mutans* and *S. sanguis* that can be mutually antagonistic (Hamada and Ooshima, 1975; Kolstad, 1976).

In another study (Lehner *et al.*, 1975c), the greatest reduction in the proportion of *S. mutans* was found in crevicular fluid and in the plaque adjacent to the crevicular fluid, whereas no significant difference was found in samples of saliva. In this study, the previous findings were confirmed, and a reduction in the number of *S. mutans* in saliva of immunised animals was also detected. The principal site of colonisation of *S. mutans* appears to be the smooth surfaces of the teeth (Carlsson, Soderholm and Almfeldt, 1969), although fissures may also harbour large numbers of the organism (Ikeda and Sandham, 1971). Saliva is not thought to be a major habitat of *S. mutans* (Gibbons and van Houte, 1975) and its presence in saliva in this study may perhaps reflect shedding

from plaque. This is supported by the fact that *S. mutans* could not be detected in saliva until it had been present in cervical plaque and fissures for several weeks.

The mechanism of the reduction in colonisation by *S. mutans* in immunised animals has not been established, and the possibilities have been discussed elsewhere (Lehner *et al.*, 1975c). It may be mediated by either serum or salivary antibodies. In this study, however, no significant difference between immunised and control animals in the salivary haemagglutinating antibody titre to HACS was detected, but significant differences in serum titres of complement-fixing and haemagglutinating antibody were maintained throughout the duration of the experiment. This suggests that serum antibodies may have played a more important role than salivary antibodies in reducing the relative number of *S. mutans* and the development of caries in this experiment. The finding of a reduced proportion of *S. mutans* in crevicular-fluid washings throughout the period of this study suggests that serum antibodies were acting at this site.

The rise in serum haemagglutinating antibodies to HACS in the control animals after 30 weeks was of interest. This may have been due to "natural" immunisation with *S. mutans*, because large numbers of this organism were present by that date. A rise in serum antibodies was also noted by Taubman (1973) in control animals given injections of Freund's complete adjuvant. An increase in serum antibodies to antigens from *S. mutans* has been found in association with the development of caries in man (Challacombe and Lehner, 1976).

In this study we examined the relationship between the presence of extra-cellular-polysaccharide-producing streptococci and the development of dental caries in rhesus monkeys, without resorting to gnotobiotic techniques or the artificial implantation of micro-organisms. The natural development of dental bacterial plaque was therefore influenced only by the diet and immunisation, but cariogenic organisms other than *S. mutans* may also have played a role in the initiation and progression of caries. The reduction in both the number of carious cavities and of *S. mutans* resulting from immunisation with *S. mutans* argues in favour of this organism playing a major part in the development of caries in these animals.

SUMMARY

In a serial investigation of the effects of immunisation with *S. mutans* in rhesus monkeys maintained on a "human" type of cariogenic diet, the numbers of *S. mutans* in cervical plaque, crevicular-fluid washings, fissures of teeth, and in saliva were lower in immunised animals than in sham-immunised controls. Immunisation also caused a delay in initial colonisation and a slowing of the rate of colonisation with *S. mutans*. These bacteriological changes were associated with a reduction in the smooth-surface-caries score. No relationship was found between the presence of *S. sanguis* and caries, but there was an inverse relationship between the proportions of *S. mutans* and *S. sanguis* isolated. Increased titres of complement-fixing, haemagglutinating and precipitating antibodies to *S. mutans* were found in the sera of immunised but not of control

monkeys. A significant increase in salivary haemagglutinating antibodies was not detected. The results suggest that immunisation with *S. mutans* causes an increase in serum antibodies and a reduction in the number of *S. mutans* in the oral flora, and that these are associated with a reduction in dental caries.

We thank Dr R. M. McKinney for supplying the conjugated *S. mutans* antisera, Mr R. G. Ward for carrying out the fluorescent-antibody tests and Dr M. W. Russell for the preparation of the HACS antigen. This work was carried out under a project grant from the Medical Research Council.

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