IMMUNISATION OF RHESUS MONKEYS WITH
STREPTOCOCCUS MUTANS, LACTOBACILLUS ACIDOPHILUS
AND LIPOTEICHOIC ACID FOR PROTECTION AGAINST
DENTAL CARIES

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SUMMARY. An attempt was made to protect rhesus monkeys from dental caries by immunisation with *Streptococcus mutans*, *Lactobacillus acidophilus* and lipoteichoic acid (LTA). The vaccine composed of *S. mutans* gave significant protection against caries, a decrease in the number of *S. mutans*, an increase in IgG antibodies and a moderate increase in complement-fixing antibodies to LTA. When LTA was used as immunogen, there was only a small reduction in caries, without any detectable antibodies to LTA and a slight increase in IgG antibodies to cells of *S. mutans*. Vaccines of *L. acidophilus* or *L. fermentum* gave no protection. A combined vaccine of *S. mutans* and *L. acidophilus* did not reduce the incidence of caries but the antibody titre to cells of *S. mutans* was raised to a level comparable with that in the *S. mutans*-immunised monkeys. The results of this investigation in a subhuman primate confirm that immunisation with *S. mutans* induces protection against caries, unlike the attempt to immunise with two selected strains of lactobacilli. More studies are required to establish the role of specific serotypes of lactobacilli in the development of dental caries.

INTRODUCTION

Lactobacilli were among the first oral micro-organisms to be isolated from carious lesions. As early as 1903 Goadby described a gram-positive rod, *Bacillus necrodentalis*, which was frequently isolated from carious dentine. Subsequently a relationship was found between salivary *Lactobacillus acidophilus* counts and caries (Jay, Hadley and Bunting, 1936; Green and Dodd, 1956). However, Ikeda, Sandham and Bradley (1973) observed that caries often occurred in the absence of lactobacilli but not in the absence of *Streptococcus mutans*. In contrast, Carlsson, Grahñen and Jonsson (1975) found that *L. casei* was the most prominent species of lactobacillus in the mouth of children, with a significantly increased number in children with caries; *S. mutans* was also isolated by them but a significant relationship with caries was not found. The number of *S. mutans*, and to a lesser extent that of lactobacilli, increases after the development of caries (Hardie et al., 1977).

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Unlike the doubtful association of lactobacilli with caries, there is little doubt that *S. mutans* is one of the most important organisms in the development of caries (Fitzgerald and Keyes, 1960; Krasse *et al*., 1968). It should be noted, nevertheless, that immunisation with *S. mutans* rarely prevents completely the development of caries. Significant but incomplete protection has been recorded in rats (Taubman and Smith 1974; Michalek *et al*., 1976), *Macaca fascicularis* (Bowen *et al*., 1975) and *Macaca mulatta* (Lehner, Challacombe and Caldwell, 1975c). Lipoteichoic acid (LTA) is found in streptococci and in lactobacilli (Knox and Wickham, 1978) and the possibility was considered that LTA is an antigen common to *S. mutans* and lactobacilli and is effective in protection against caries. We have therefore attempted to protect monkeys from caries by immunisation with lactobacilli and LTA.

**Materials and methods**

**Vaccines.** Whole cells were treated with formalin as described by Caldwell, Challacombe and Lehner (1977). The *S. mutans* was of serotype c (Guy's strain), isolated from a child with rampant caries. The *L. acidophilus* was isolated from a rhesus monkey maintained on a "human" diet. The LTA was prepared from *S. mutans* (serotype c, Guy's strain) by phenol extraction (Wicken, Gibbens and Knox, 1973) and was complexed with methylated bovine serum albumin (MeBSA) as described by Fiedel and Jackson (1976).

**Immunisation of animals.** A total of 18 young rhesus monkeys (*Macaca mulatta*) with fully erupted deciduous teeth were randomly distributed into five groups. Three monkeys received $5 \times 10^8$ formalinised whole cells of *S. mutans* in Freund's incomplete adjuvant (FIA); two others received $5 \times 10^8$ formalinised whole cells of *L. acidophilus* in FIA; three received a combined vaccine of $2.5 \times 10^8$ cells of *S. mutans* with $2.5 \times 10^8$ cells of *L. acidophilus*; and three were given injections of 1 mg of LTA/MeBSA complex in FIA. A control group of seven monkeys were sham-immunised with 1 ml of physiological saline.

The vaccines were injected subcutaneously in two equally divided doses into opposite upper and lower limbs. A second injection of vaccine without FIA was administered 12–16 weeks after the primary immunisation. The animals were maintained on a "human" high-sucrose diet, they were examined at two-monthly intervals and the caries score was determined as described by Lehner, Challacombe and Caldwell (1975a).

**Collection of specimens.** Samples of plaque were collected from the cervical margins and approximal surfaces of the upper left first and second deciduous molars with a sterile dental probe. A second sample of plaque was taken from the fissures of the same teeth. Samples of blood were also collected at two-monthly intervals (Lehner, Challacombe and Caldwell, 1975b).

**Bacteriological methods.** The samples of plaque were transferred immediately to transport medium (Bowden and Hardie, 1971) then mixed vigorously with sterile glass beads. Three tenfold dilutions were made in transport medium and 0.1 ml of each dilution was inoculated on duplicate plates of blood agar (Oxoid), TYC medium (de Stoppelaar, van Houte and de Moor, 1967) and lactobacillus medium (Rogosa, Mitchell and Wiseman, 1951). The plates were incubated for 4 days at 37°C in an atmosphere of $H_2 + 5\% \ (v/v) CO_2$. A total count of colony-forming units (cfu) was made on the blood-agar plates. *S. mutans* was identified and counted on TYC medium, as described by Caldwell *et al.* (1977). The colonies grown on lactobacillus medium that were catalase-negative gram-positive rods were counted and subcultured. Species of lactobacilli were identified by the methods of Davis (1955), Hayward and Davies (1956) and Hayward (1957). The numbers of *S. mutans* and lactobacilli were expressed as percentages of the total anaerobic counts on blood agar, and the numbers of *S. mutans* as the percentage of the total count on TYC medium.

**Serological methods.** Serum IgG antibody titres were determined by the indirect immunofluorescence method, from air-dried smears of *S. mutans* and *L. acidophilus* (Lehner *et al*., 1979). A specific rabbit antihuman IgG fluorescein conjugate (F: P ratio 3:1, Wellcome Reagents) was used; its specificity was determined as described by Lehner *et al.* (1976). Complement-fixing (CF) serum antibodies against *L. acidophilus* cell wall and LTA were assayed as described by
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Caldwell et al. (1977). The cell-wall antigen was prepared by the method of Challacombe (1974) but without enzyme treatment.

RESULTS

Caries scores

The sequential development of caries is shown in fig. 1. Fifty-six weeks after primary immunisation with S. mutans, the monkeys had a low mean caries score (±SE) of 2.6 (±2.3) compared with the control animals 11.8 (±3.8). The development of caries in the L. acidophilus group of monkeys was very similar to that of the control group and at 56 weeks had reached a score of 11. The animals that had received the combined streptococcus and lactobacillus vaccine and the LTA-vaccinated animals had very similar caries scores at 56 weeks of 9.0 (±2.1) and 7.7 (±0.7), respectively. The LTA-immunised monkeys had a score that was intermediate between those of the S. mutans-immunised and control groups, but there was no significant protection.

Culture of S. mutans

The sequential counts of S. mutans in smooth-surface plaque (table I) showed that fewer S. mutans were isolated from monkeys immunised with S. mutans than from all other groups. This result was obtained in four out of seven serial samples, when expressed as a percentage of the total count on either TYC or blood agar. The fissure plaque in the same monkeys (table II) showed a similar trend, four out of seven samples on TYC agar, but only two samples on blood agar. Caries first appeared in the

![Fig. 1—Mean caries scores (Lehner et al., 1975a) ± SE in monkeys immunised with S. mutans (●—●), L. acidophilus (●—●), combined vaccine (■—■), lipoteichoic acid (▲—▲) or saline (○—○).]
<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>0 weeks on</th>
<th>8 weeks on</th>
<th>16 weeks on</th>
<th>24 weeks on</th>
<th>32 weeks on</th>
<th>40 weeks on</th>
<th>48 weeks on</th>
<th>56 weeks on</th>
</tr>
</thead>
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<tr>
<td></td>
<td>TYC</td>
<td></td>
<td>TYC</td>
<td></td>
<td>TYC</td>
<td></td>
<td>TYC</td>
<td></td>
</tr>
<tr>
<td>S. mutans</td>
<td>2.3</td>
<td>0.1</td>
<td>4.3</td>
<td>37.0</td>
<td>62.0</td>
<td>21.0</td>
<td>62.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Combined vaccine</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>L. aridophilus</td>
<td>0.1</td>
<td>10.7</td>
<td>10.7</td>
<td>10.7</td>
<td>10.7</td>
<td>10.7</td>
<td>10.7</td>
<td>10.7</td>
</tr>
<tr>
<td>Lipoteichoic acid</td>
<td>10.7</td>
<td>10.7</td>
<td>10.7</td>
<td>10.7</td>
<td>10.7</td>
<td>10.7</td>
<td>10.7</td>
<td>10.7</td>
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<tr>
<td>Control</td>
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<td>10.7</td>
<td>10.7</td>
<td>10.7</td>
<td>10.7</td>
<td>10.7</td>
<td>10.7</td>
<td>10.7</td>
</tr>
</tbody>
</table>

TYC = TYC medium of de Stoppelaar et al. (1967); BA = blood agar; ND = not done.

*Expressed as percentage of the total anaerobic count on the same culture medium; SE is not given for means based on only two samples.

TYC medium of de Stoppelaar et al. (1967); BA = blood agar; ND = not done; SE is not given for means based on only two samples.

TABLE I

Streptococcus mutans in smooth-surface plaque

Mean number (± SE)* of colony-forming units at 0 weeks on 8 weeks on 16 weeks on 24 weeks on 32 weeks on 40 weeks on 48 weeks on 56 weeks on

TYC = TYC medium of de Stoppelaar et al. (1967); BA = blood agar; ND = not done. SE is not given for means based on only two samples.

* Weeks counted from time of primary immunisation.
### Table II

*Streptococcus mutans* in fissure plaque

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>0 weeks† on</th>
<th>8 weeks on</th>
<th>16 weeks on</th>
<th>24 weeks on</th>
<th>32 weeks on</th>
<th>40 weeks on</th>
<th>48 weeks on</th>
<th>56 weeks on</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TYC</td>
<td>BA</td>
<td>TYC</td>
<td>BA</td>
<td>TYC</td>
<td>BA</td>
<td>TYC</td>
<td>BA</td>
</tr>
<tr>
<td><em>S. mutans</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Combined vaccine</td>
<td>0.67</td>
<td>&lt;0.1</td>
<td>22.7</td>
<td>13.5</td>
<td>24.0</td>
<td>28.5</td>
<td>19.7</td>
<td>1.0</td>
</tr>
<tr>
<td><em>L. acidophilus</em></td>
<td>0.33</td>
<td>0.3</td>
<td>49.0</td>
<td>33.5</td>
<td>16.3</td>
<td>4.0</td>
<td>8.3</td>
<td>3.7</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>27.3</td>
<td>18.0</td>
<td>62.0</td>
<td>30.5</td>
<td>24.0</td>
<td>15.0</td>
</tr>
</tbody>
</table>

Footnotes as in Table I.
sham-immunised monkeys at 16 weeks and the numbers of *S. mutans* at this time in three groups are shown in fig. 2. The counts of *S. mutans* were lower in the *S. mutans*-immunised than in the LTA-immunised or control monkeys.

**Culture of lactobacilli**

The sequential counts of lactobacilli are shown in tables III and IV. They formed a much smaller percentage of the total anaerobic count than those of *S. mutans* or the sham-immunised monkeys. However, fewer lactobacilli were found in the *S. mutans*-immunised monkeys in six out of the seven samples of smooth-surface plaque but in only three samples of fissure plaque. Lactobacillus counts were not done on the LTA-immunised animals. A total of 556 lactobacillus colonies were subcultured for species identification. *L. fermentum* (46.2%) was the most common; other species were *L. acidophilus* (19.7%), *L. cellobiosis* (17.8%), *L. casei* (4.2%), *L. salivarius* (1.0%) and unidentified (11.1%).

**Antibodies to *S. mutans* and *L. acidophilus***

The control and *L. acidophilus*-immunised animals had no detectable IgG
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TABLE III
Lactobacilli in smooth surface plaque

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>Mean number (± SE)* of colony-forming units at</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 weeks† 8 weeks 16 weeks 24 weeks 32 weeks 40 weeks 48 weeks 56 weeks</td>
</tr>
<tr>
<td>S. mutans</td>
<td>0.19 0.01 0.03 0.20 0.07 0.03 0.14 0.05</td>
</tr>
<tr>
<td>Combined vaccine</td>
<td>(±0.20) (±0.32) (±0.01) (±0.30) (±0.17) (±0.05) (±0.04) (±0.70)</td>
</tr>
<tr>
<td>L. acidophilus</td>
<td>0.35 0.20 0.51 0.30 0.15 1.31 0.06 0.18</td>
</tr>
<tr>
<td>Control</td>
<td>0.07 0.12 0.13 0.70 0.37 0.35 3.99 0.06</td>
</tr>
<tr>
<td></td>
<td>(±0.03) (±0.05) (±0.40) (±0.16) (±0.23) (±3.70) (±0.04) (±0.05)</td>
</tr>
</tbody>
</table>

*† As in table I.

TABLE IV
Lactobacilli in fissure plaque

<table>
<thead>
<tr>
<th>Vaccine group</th>
<th>Mean number (± SE)* of colony-forming units at</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 weeks† 8 weeks 16 weeks 24 weeks 32 weeks 40 weeks 48 weeks 56 weeks</td>
</tr>
<tr>
<td>S. mutans</td>
<td>28.0 2.0 1.5 0.1 16.5 1.8 123 19.0</td>
</tr>
<tr>
<td>Combined vaccine</td>
<td>37.0 0.3 5.0 22.7 0.2 12.3 3.5 19.7</td>
</tr>
<tr>
<td>L. acidophilus</td>
<td>4.0 22.5 5.8 24.0 1.6 5.7 1.7 4</td>
</tr>
<tr>
<td>Control</td>
<td>2.8 3.0 4.6 7.9 2.4 7.9 4.3 5.4</td>
</tr>
<tr>
<td></td>
<td>(±1.4) (±1.6) (±1.9) (±5.7) (±1.3) (±3.8) (±1.8) (±2.4)</td>
</tr>
</tbody>
</table>

*† As in table I.

antibodies to S. mutans by the indirect immunofluorescence test (fig. 3). The LTA group had a modest response, a mean titre of log₂ 1.6 (± 1) at 16 weeks rising to log₂ 2.0 (± 1) at 48 weeks. The monkeys that received the S. mutans or the combined vaccine had strong IgG responses, log₂ 8.0 ± 0 and 6.5 ± 0.6 respectively, at 16 weeks. These titres were well maintained, dropping only to log₂ 5.7 (± 1.2) in the S. mutans and to log₂ 5.6 (± 1.2) in the combined-vaccine group 56 weeks after immunisation.

In contrast, the response to immunisation with L. acidophilus was poor. In the L. acidophilus-immunised animals the titre reached only log₂ 3.0 at 8 weeks, which dropped to log₂ 1.5 at 40 weeks. Like the antibodies to S. mutans, those in the combined-vaccine group showed a slightly lower titre, log₂ 2.0 (± 1.5) at 8 weeks, falling to 0.7 (± 0.7) at 40 weeks. The control, S. mutans and LTA-immunised animals had no detectable IgG antibodies to L. acidophilus.

Most of the animals had detectable levels of CF antibodies to L. acidophilus cell wall before immunisation. The highest response was in the monkeys immunised with S. mutans (fig. 4). Monkeys immunised with L. acidophilus showed a poor response, with titres of log₂ 2.5 at 8 weeks. The titres of CF antibodies failed to increase by immunisation with the combined vaccine or LTA, though the pre-immune titres were somewhat elevated.
CF antibodies to LTA were not detected before immunisation. Although the response was weaker, as was seen also with the *L. acidophilus* cell-wall antigen, the *S. mutans*-immunised animals again elicited the highest response, with titres of log$_2$ 3.3 (±0.3) at 8 weeks. The animals immunised with LTA had no detectable CF antibodies to LTA. The *L. acidophilus* and control animals had negligible titres and at 8 weeks the monkeys immunised with the combined vaccine had low titres of CF antibodies to LTA, log$_2$ 1.7 (±1.2).

DISCUSSION

Immunisation with cells of *S. mutans* induced significant protection against dental
Fig. 4—Mean titre ± SE of complement-fixing antibody to lipoteichoic acid (A) and L. acidophilus cell walls (B) in monkeys immunised with S. mutans (●—●), L. acidophilus (○—○), combined vaccine (■——■), lipoteichoic acid (▲——▲) or saline (○——○).
S. mutans-immunised monkeys developed the highest titres of antibodies not only to S. mutans but also to LTA. Although the titres of CF antibodies to LTA were rather low, about 20, their presence is consistent with LTA playing some part in the protection against caries. The potential biological importance of LTA has been reviewed by Knox and Wickham (1978) and its significance in the development of caries by Rolla (1980). LTA might be particularly involved in the early stages of colonisation of cariogenic organisms. In our study, monkeys immunised with LTA showed intermediate values in caries scores, antibodies and the number of colonies of S. mutans just after the onset of clinical caries, at 16 weeks.

The combined S. mutans–L. acidophilus vaccine was tried out with the view of increasing the 60–80% reduction in caries achieved in rhesus monkeys (Lehner et al., 1975c, 1976; Caldwell et al., 1977). However protection was not achieved, despite good antibody titres to S. mutans. This ambiguous result will have to be investigated further and the interpretation that might be pursued is that there may be interference or competition between antibodies to streptococci and lactobacilli. This might be especially critical in the crevicular domain, where gingival-fluid antibodies are diluted by saliva (Challacombe et al., 1978; Smith and Lehner, 1981).

Quantitative analysis of S. mutans in the five groups of monkeys suggests that only the S. mutans-immunised monkeys show a lower incidence of this organism in plaque from smooth surfaces and from fissures, when compared with the controls or any of the other immunised groups of monkeys. Surprisingly, the incidence of lactobacilli was also generally lower in this same group of immunised monkeys. One explanation is that the lactobacilli, being aciduric, thrive in the acid environment created by S. mutans. Hence, a decrease in S. mutans might be accompanied by a decrease in the lactobacilli.

L. fermentum was the most common species. It is therefore possible that the most appropriate lactobacillus was not chosen for the vaccine. We have since attempted to immunise two monkeys with $5 \times 10^8$ L. fermentum in FIA, but this did not result in any obvious protection against caries. This makes it less likely that lactobacilli play an important part in the development of caries in rhesus monkeys. Unlike S. mutans, of which group c is the most common serotype in man (Bratthall and Köhler 1976) and in rhesus monkeys (unpublished data), little is known about the serotypes of species of lactobacilli and their role, if any, in dental caries. Serological groups among lactobacilli (Sharpe, 1955, 1970) correspond to physiologically identifiable species. It should be emphasised that Fitzgerald (1963) was unable to induce caries in gnotobiotic rats with either L. acidophilus or L. fermentum, though a variant of L. acidophilus produced fissure caries (Fitzgerald, Jordan and Archard, 1966).

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REFERENCES


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