Antibody Responses to Antigens of *Streptococcus mutans* in Monkeys (*Macaca fascicularis*) Immunized against Dental Caries

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(Received 23 August 1982)

Immunization of rabbits or monkeys with walls prepared from *Streptococcus mutans* by a procedure including extraction with SDS at room-temperature induced antibodies to three antigens (A, B and C) detectable by crossed immunoelectrophoresis. Antigens A and B have previously been characterized as proteins of molecular weight 29 000 and 190 000, respectively. Antigen C was characterized as having a molecular weight of 70 000 and was purified by immunosorbent affinity chromatography and hydrophobic interaction chromatography. Another wall protein, antigen D, of molecular weight 13 000, was extracted from walls with Triton X-100. Immunization of monkeys with walls prepared from cultures of *S. mutans* grown at a high ($D = 0.5 \text{ h}^{-1}$) or low ($D = 0.05 \text{ h}^{-1}$) dilution rate in a chemostat showed that only the latter induced protection against dental caries. There was a positive correlation between levels of antibody to antigens A and C and induction of protection and a negative correlation between protection and the level of antibody to antigen B. No antibody to antigen D was detected in protected monkeys and an experiment in which monkeys were immunized with pure antigen D confirmed that it does not induce protection.

INTRODUCTION

Streptococcus mutans is believed to be of prime significance in dental caries and reports from a number of laboratories have described the successful immunization of experimental animals against the disease with vaccines based on *S. mutans* (reviewed by McGhee & Michalek, 1981). Experiments involving monkeys have indicated that a protein component of the wall of *S. mutans* is essential for protection (Colman & Cohen, 1979; Russell *et al.*, 1979) and we have previously described wall-associated protein antigens A and B of *S. mutans* serotype *c* and their use as purified vaccines (Russell, 1979*a, b*; Russell *et al.*, 1982). Antigens A and B were identified as proteins which remain associated with walls even after extraction with the detergent SDS at 100 °C. Walls prepared by more gentle methods can also confer protection against caries (Bowen *et al.*, 1975; Cohen *et al.*, 1979) and the possibility existed that such wall preparations contained potentially protective antigens in addition to A and B. Cohen *et al.* (1979) also described an experiment in which monkeys were immunized with walls prepared from *S. mutans* grown at different rates in a chemostat and presented preliminary data showing that a slow growth rate resulted in walls conferring most protection. Those preliminary data are extended in the present paper which describes the antigenic analysis of the protein component of *S. mutans* walls, the characterization and purification of two new antigens (C and D) and an analysis of immune response and caries development in immunized monkeys.

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Some of the results were presented at the VIIIth International Symposium on Streptococci and Streptococcal Diseases in June 1981 (Peach & Russell, 1982) and at the 94th Ordinary Meeting of the Society for General Microbiology in Cambridge, April 1982.

METHODS

Organisms and growth conditions. The following strains, representing *Streptococcus mutans* serotypes *a* to *h*, were used: AHT and OMZ-61 (*a*); FA1 and OMZ-52 (*b*); Ingbritt, NCTC 10449, FW293, GS-5, OMZ-70, C67-1, 3209 (*c*); B13 (*d*); P4 (*e*); 151 (*f*); K1 (*g*); MFe 28 (*h*).

For the preparation of large volumes of culture, Tryptone-yeast extract broth (Tryptone 10 g; yeast extract, 3 g; NaCl, 2 g; K_2HPO_4 , 2 g; $NaHCO_3$, 1 g; glucose, 5 g; distilled water, 1 l) was used with the pH maintained at 6.5 by the automatic addition of KOH. Extracellular proteins were also prepared from the supernatant of cultures grown in a semi-defined medium consisting of Casamino acids with added salts, vitamins and bases (Russell, 1979c).

Chemostat growth of *S. mutans* Ingbritt used to prepare vaccines for experiment 19 (see below) was kindly undertaken by M. Hughes of the Wellcome Research Laboratories using the medium and conditions described by Ellwood *et al.* (1974). In later experiments *S. mutans* was grown in a 1 l fermentation vessel (Series 500, L. H. Engineering, Stoke Poges, Bucks.) at 37 °C in the medium described by Wenham *et al.* (1979) with the addition of cysteine hydrochloride (1 g l⁻¹) and glucose (6 g l⁻¹). This concentration of glucose was growth-limiting.

Preparation of SDS-washed walls. *Streptococcus mutans* grown as described above was heated at 60 °C for 30 min to inactivate autolytic enzymes and washed twice by centrifugation in 0.15 M-NaCl. The bacteria were then broken by shaking with glass beads (Ballotini no. 12) in a Mickle tissue disintegrator for 20 min or in a Braun cell homogenizer for 3 min. Broken bacteria were separated from the beads with a sintered glass filter funnel and washed twice by centrifugation with distilled water, twice with 1 M-NaCl and then resuspended in 1% (w/v) SDS at room temperature with the aid of a glass/Teflon tissue grinder. Intact bacteria were removed by centrifugation at 3000 g for 20 min. The walls were sedimented by centrifugation at 11 000 g for 20 min. The walls were again resuspended in SDS and the differential centrifugation was repeated. Finally walls were washed three times in distilled water and freeze-dried.

Mutanolysin digestion of walls. Mutanolysin M-1 enzyme was generously provided by M. Yokogawa (Yokogawa *et al.*, 1975). SDS-washed walls were suspended at a concentration of 20 mg ml⁻¹ with 200 µg M-1 ml⁻¹ in 0.01 M-Tris/maleate/NaOH buffer (pH 7.0) and mixed overnight at 37 °C. Insoluble material was removed by centrifugation and discarded.

Preparation of antisera in rabbits. Injection schedules consisted of two or more intramuscular injections of 1 mg walls or 50 to 100 µg pure antigen in 0.9 ml phosphate-buffered saline containing 0.1 ml aluminium hydroxide (Serva Alu Gel S, Uniscience, Cambridge) at 3-weekly intervals. Rabbits were bled 1 week after a booster and the immunoglobulin fractions of antisera were prepared by either the method of Closs *et al.* (1975) or Steinbuch & Audran (1969).

Electrophoretic techniques. Methods for rocket and crossed immunoelectrophoresis, SDS-PAGE and isoelectric focusing have been described previously (Russell, 1979b). Crossed immunoelectrofocusing was also performed in agarose by the method of Rosen *et al.* (1979). For two-dimensional electrophoresis the procedure of O'Farrell (1975) was followed, except that agarose was used for the stacking gel in the second dimension (Aasted, 1980).

Proteins were transferred electrophoretically from acrylamide gels to nitrocellulose and probed with antiserum ('Western blotting') by published procedures (Towbin *et al.*, 1979; Burnette, 1981). Antigens to which antibody had bound were located with staphylococcal Protein A (Sigma) radioiodinated by the method of Erlich *et al.* (1978) followed by autoradiography or by peroxidase-conjugated swine antiserum to rabbit immunoglobulins (Dako, Mercia Brocades, Weybridge) and subsequent incubation with 3-amino-9-ethyl carbazole (Karcher *et al.*, 1981).

Experiments with radio-labelled antigens. Antigen preparations were labelled with ¹²⁵I by use of 1,3,4,6-tetrachloro-3α,6α-diphenyl glycouril-coated beads (Iodo-beads, Pierce & Warriner Ltd, Chester). Antigen preparation (100 µl containing approximately 100 µg total protein) was placed in a tube with a single Iodo-bead and 50 µCi Na¹²⁵I (1.85 MBq; Amersham). After 15 min at room temperature the reaction was terminated by removing the bead and adding 10 µl 2.5 M-NaI as carrier.

Identification of antigens in individual immunoprecipitates was performed essentially as described by Norrild *et al.* (1977): 20 µl of an ¹²⁵I-labelled crude antigen pool was subjected to crossed immunoelectrophoresis on a 5 × 5 cm slide with antiserum raised against SDS-washed walls. The slide was washed, dried and stained in the same manner as for non-radioactive slides then placed in water for 5 min to soften the agarose. Non-overlapping regions of each stained precipitin peak were excised with a scalpel blade and transferred to sample buffer containing SDS for solubilization prior to PAGE. Gel slabs were dried on Whatman 3 MM filter paper using a Bio-Rad Model 224 drier (Bio-Rad) and autoradiographs were prepared by exposing dried gels to Kodak X-Omat AR film with Du Pont Lightning Plus intensifying screens at -20 °C (Swanstrom & Shank, 1978).

Immunoprecipitation experiments in which radiolabelled antigen/antibody complexes were collected on Protein A-bearing staphylococci (Pansorbin, Calbiochem) for analysis by SDS-PAGE used methods described by Kessler (1981).

Purification of antigen C. Culture filtrate of *S. mutans* strain Ingbritt was brought to 65% saturation at 4 °C with ammonium sulphate. The precipitate which formed overnight was collected by centrifugation and redissolved in 0.05 M-Tris/HCl buffer (pH 7.5) at a concentration of approximately 1 mg ml⁻¹ before being passed through a chromatography column containing 9 ml Sepharose CL-6B (Pharmacia) and 500 mg mutan. Mutan is the water-insoluble glucan produced from sucrose by *S. mutans* and was prepared as described by Russell (1979*d*). This affinity column removed the bulk of the glucan-binding proteins which would otherwise be retained non-specifically by subsequent immunosorbent columns (Russell, 1981). Two procedures for purification of antigen C have been used. In the first, briefly described elsewhere (Peach & Russell, 1982), the antigen preparation after passage through the mutan/Sepharose column was passed through a series of immunosorbent columns prepared by coupling the IgG fraction of serum from immune rabbits to cyanogen bromide-activated Sepharose (Pharmacia). The columns contained (1) monospecific antibody to antigen A, (2) monospecific antibody to antigen B, (3) antibody to SDS-washed walls (i.e. antibody to antigens A, B and C). Preparation and use of immunosorbent columns was as described by Russell (1981). Antigens A and B were retained by the first two immunosorbent columns and hence only antigen C was bound to the final column and could be eluted from it by 3 M-sodium thiocyanate in 0.05 M-Tris/HCl pH 7.5. Fractions containing antigen were pooled, after detection by rocket immunoelectrophoresis into antiserum to SDS-washed walls.

In the second procedure, eluate from the mutan/Sepharose column was passed through the immunosorbent column containing antibody to SDS-washed walls. After extensive washing of the column with Tris buffer, antigens A, B and C were eluted with thiocyanate as above. The fractions of eluate which contained antigen were dialysed first against 0.05 M-Tris/HCl pH 7.5 and then against the same buffer containing 1 M-ammonium sulphate. The antigen sample was then applied to a chromatography column containing Phenyl-Sepharose CL-4B (Pharmacia). Proteins retained by the column were eluted by a gradient of decreasing ammonium sulphate concentration and concurrently increasing ethylene glycol concentration [final concentration 0 and 50% (v/v), respectively]. Antigen C was eluted between 0.6 and 0.7 M-ammonium sulphate/15 to 20% (v/v) ethylene glycol in advance of antigens A, B and any residual glucan-binding proteins.

Purification of antigen D. Lyophilized *S. mutans* walls (approximately 20 mg ml⁻¹) prepared as described above were extracted with 1% Triton X-100 in 0.05 M-Tris/HCl pH 7.5 on a stirrer overnight at room temperature. Insoluble material was removed by centrifugation and the extract was applied to a column containing DEAE-Sephadex A25 (Pharmacia) equilibrated with 0.05 M-Tris/HCl pH 7.5. After extensive washing to remove all detergent, the column was eluted with a gradient of 0 to 0.3 M NaCl in 0.05 M-Tris/HCl pH 7.5 and fractions were collected and examined by SDS-PAGE for their content of antigen D. Fractions containing antigen were pooled, concentrated by lyophilization and applied to a column of Bio-Gel P-150 (Bio-Rad). Fractions containing antigen eluted from the column were again detected by SDS-gel electrophoresis and pooled. Throughout the purification the presence of other wall antigens was monitored by SDS-gel electrophoresis and rocket immunoelectrophoresis into antiserum raised against walls.

Antigen contents of other serotypes. The collection of strains of *S. mutans* was examined for the presence of different antigens by testing for reaction with rabbit antisera specific for each antigen (A, B, C and D) in the following tests: (1) immunodiffusion of concentrated culture supernatants or mutanolysin-digested bacteria (Russell, 1980); (2) agglutination of cultures either on slides or in U-well microtitration trays containing a standardized bacterial suspension and serially-diluted antiserum; (3) a procedure in which microtitration tray wells were coated with whole bacteria and the extent to which antiserum bound was assessed by ELISA (Russell & Beighton, 1982).

Monkey immunization experiments. Three separate experiments involving *Macaca fascicularis* monkeys are described. Details of the earliest experiment including the source of the animals, their diets and dental caries experience have been described as experiment C of Bowen *et al.* (1975) and Cohen *et al.* (1979).

Experiment 19 was briefly described by Cohen *et al.* (1979) and further details of diet composition and methods for bacteriological examination of dental plaque were presented by Beighton & Hayday (1982). At the start of the experiment groups of monkeys immunized with whole bacteria were included (Cohen *et al.*, 1979) but these groups had to be discarded because of several incidental deaths among the animals. The groups described in the present report were: (1) a control group of five monkeys (three males and two females), mean age 9.4 months, mean weight 1.34 kg at the start of the experiment; (2) four monkeys (three males and one female, mean age 9.75 months, mean weight 1.42 kg) which were immunized with walls prepared from *S. mutans* grown in a chemostat at $D = 0.5 \text{ h}^{-1}$; (3) three monkeys (two males and one female, mean age 11.3 months, mean weight 1.28 kg) which were immunized with walls from *S. mutans* grown in a chemostat at $D = 0.05 \text{ h}^{-1}$. The immunization schedule used in experiment 19 was arranged to resemble that used earlier in experiment C of Bowen *et al.* (1975), i.e. multiple injections totalling 0.5 mg walls in sterile saline were made into the mucosal surfaces of the mouths. Nine

injections were administered at 2-weekly intervals prior to exposure to the caries-promoting sucrose-rich (CHS) diet. Booster injections were administered by the same route 7, 11, 14, 23 and 38 months after the start of the experiment.

Experiment 23 differed from earlier experiments in that the animals were housed in two large communal groups instead of singly or in pairs. The animals in this experiment have also been the subject of a bacteriological study (Group 2 described in Beighton & Hayday, 1982). When immunization was commenced, the animals were assigned as controls (four males, two females, mean age 9.8 months, mean weight 1.2 kg), a group immunized with pure antigen D (four males, two females, 9.0 months, 1.15 kg) and a group immunized with walls which had been digested with mutanolysin (three males, one female, 8.0 months, 1.14 kg). For immunization with antigen D or wall-digest, monkeys were injected with 50–100 µg protein in sterile solution with 10% (w/v) aluminium hydroxide adjuvant at two subcutaneous sites in the lower limbs. Injections were given at the start of the experiment and 1, 3 and 12 months thereafter.

Methods of blood sampling and procedures for assay of antibody levels by ELISA were as described by Russell & Colman (1981).

RESULTS

Rabbits immunized with *S. mutans* Ingbritt which had been extracted with SDS at 100 °C produced antibodies to only two protein antigens, A and B (Russell, 1979*a, b*). In contrast walls washed in SDS at room temperature as described in Methods induced antibody to three antigens which could be detected by crossed immunoelectrophoresis (Fig. 1). The same three antigens were present in mutanolysin-digests of walls and in culture supernatant preparations and so must exist in a wall-associated form which resists removal with SDS, as well as being released into the growth medium. Of a total of eight rabbits immunized with walls, all developed antibody to the same three antigens, though the relative levels of antibody to each antigen depended on the source of the walls used for immunization (see below).

The new antigen C was destroyed by pronase, indicating that it was a protein. Its physical characteristics were determined by crossed immunoelectrophoresis in which the first dimensional separation was by SDS-PAGE or by isoelectric focusing in polyacrylamide or agarose. The results obtained indicated that antigen C had a molecular weight of 70000 and an isoelectric point of 4.45.

The size of antigen C was also determined in another experiment in which an ¹²⁵I-labelled antigen preparation was subjected to crossed immunoelectrophoresis against antiserum to walls. The radioactive immunoprecipitates were solubilized in SDS and the antigens in each precipitate identified by slab gel electrophoresis followed by autoradiography. Non-radioactive molecular weight standards were used on the same slab to allow determination of the size of the antigens. The results (Fig. 2) confirmed the size of the antigen C as 70000. It can also be seen that the antigen B precipitin line contained not only intact B (mol. wt 190000) but many smaller fragments as well.

Purification of antigen C

Antigen C purified from the supernatant of a culture in the semi-defined medium by either of the procedures in Methods gave a single band on SDS-PAGE or isoelectric focusing in acrylamide. The size and charge of the pure molecule were the same as already determined by indirect means with unpurified material. The purity of antigen C was also assessed by transfer from an SDS-polyacrylamide gel slab to nitrocellulose, followed by probing with a polyvalent antiserum (i.e. the 'Western blot' technique). The antiserum used was capable of detecting at least 40 antigenic bands in crude antigen preparations, but antigen C was the only one present in the purified preparation. In order to increase the sensitivity of detection of contaminants, antigen C was labelled with ¹²⁵I and subjected to gel electrophoresis and autoradiography. No other bands besides C were detected.

Molecular weight of antigen C is close to that of a major glucan-binding protein (mol. wt. 74000, Russell, 1979*d*), fructosyltransferase (mol. wt. 70000, Russell, 1979*e*) and dextranase (mol. wt approx. 80000, H. Kuramitsu and C. W. I. Douglas, personal communications). However antigen C could be demonstrated to be distinct from each of these by their separation

Fig. 1. Crossed immunoelectrophoresis of concentrated culture supernatant proteins (50 μ g) of *S. mutans* Ingbritt against rabbit antiserum to SDS-washed walls (100 μ l in 3 ml agarose) on a 5×5 cm glass slide. Electrophoresis was at 6 V cm^{-1} for 1.5 h in the first dimension (anode to left) and 1 V cm^{-1} for 16 h in the second dimension (anode at top). The same three precipitin peaks could be detected when a mutanolysin-digested wall preparation was used as antigen.

Fig. 2. Autoradiograph of SDS-polyacrylamide gel on which the samples were precipitin peaks excised from a crossed immunoelectrophoresis slide like that shown in Fig. 1, but with ^{125}I -labelled antigen. Molecular weights of antigens A, B and C are indicated on the left hand side of the figure.

on SDS-polyacrylamide gel or isoelectric focusing; furthermore specific antiserum raised against antigen C did not precipitate or inhibit the function of any of the proteins having similar size.

Purification of antigen D

Antigens A, B and C are all released into culture supernatants. The possibility existed however that there were other wall protein antigens which were not released, but always remained bound to wall peptidoglycan. Samples of walls prepared by the SDS-washing procedure were therefore subjected to extraction with a wide range of different detergents, salts,

chaotropic agents, extremes of temperature or pH and proteolytic or muralytic enzymes. The protein composition of extracts was examined by gel electrophoresis and it was found that besides variable but low amounts of antigens A, B and C the only other band present in substantial amounts was one with a molecular weight of 13000. This was termed antigen D. While antigen D could be effectively released from walls by a number of agents, the non-ionic detergent Triton X-100 was used in the purification procedure, so that it could be separated from extracted proteins by retention of the latter on an ion-exchange column.

Antigen D was eluted from DEAE-Sephadex A25 by approximately 0.15 M-NaCl. Although SDS-PAGE suggested that antigen D eluted from the ion-exchange column was pure, it was possible to demonstrate the additional presence of antigen A by rocket immunoelectrophoresis. The traces of antigen A were removed by the final gel filtration step on Bio-Gel P150.

Antigen D preparations gave only a single band on SDS-PAGE when either gels of various acrylamide concentrations or gradient gels were used. However, isoelectric focusing in acrylamide (Wrigley, 1971) or two-dimensional gel electrophoresis (O'Farrell, 1975) revealed the presence of three or four closely spaced components of the same size but with values from pI 4.5-4.8.

Antigenicity of antigen D

Antigen D forms only very faint and non-reproducible precipitin lines in agarose (presumably because of its low molecular weight). Two methods which do not rely on the formation of immunoprecipitates in gel were therefore employed to explore interactions between antigen D and antibody. In the ELISA method, the wells of plastic microtitration trays were coated with antigen D; the extent to which rabbit or monkey antibodies bound to the immobilized antigen could then be measured by using anti-IgG conjugated to alkaline phosphatase. In the immunoprecipitation method antigen D, labelled with ^{125}I by means of Iodo-beads, was mixed with the test serum; immune complexes were collected on Protein A-containing *Staphylococcus aureus* by centrifugation and analysed by SDS-gel electrophoresis and autoradiography or by direct counting of radioactivity. The ELISA and immunoprecipitation procedures gave identical results and showed that of eight rabbits immunized with walls, only three had formed antibody to antigen D; antigen D did not react with antisera to antigens A, B or C (nor did antiserum to antigen D precipitate those antigens); pure antigen D induced a specific immune response in rabbits.

Distribution of antigens C and D amongst S. mutans serotypes

Antiserum raised against pure antigen C from *S. mutans* Ingbritt (serotype *c*) precipitated antigens in supernatants or mutanolysin extracts of all the other serotype *c* stock strains tested and also strains of serotypes *e* and *f*. The precipitated antigens from all these strains formed a line of identity with that from Ingbritt. The agglutination and ELISA techniques confirmed that antibody to antigen C bound to the surface of bacteria of serotypes *c*, *e* and *f* and also, although more weakly, to serotype *b* strains. This indicated that a cross-reacting antigen may exist in serotype *b*. There was no evidence for such cross-reactions from serotypes *a*, *d*, *g* and *h*.

The poor ability of antigen D to form precipitin lines precluded the use of immunodiffusion to screen for cross-reacting antigens but in both of the more sensitive agglutination and ELISA tests specific anti-D serum reacted strongly with all strains of serotypes *c*, *e* and *f*, though not with representatives of other serotypes.

Antibody responses in monkeys immunized with walls

In experiment C described by Bowen *et al.* (1975) and Cohen *et al.* (1979) five control monkeys were compared with four monkeys immunized with walls prepared from *S. mutans* strain Ingbritt which had been grown in a chemostat at $D\ 0.05\ \text{h}^{-1}$. Almost total protection against caries was obtained in the immunized group. Only very limited amounts of serum from the animals in this experiment are still available but we have demonstrated by crossed immunoelectrophoresis techniques that protected animals had antibody to antigens A, B and C. There was no detectable antibody to other protein antigens. ELISA confirmed that protected

Fig. 3. Progression of dental caries in the permanent teeth of monkeys in experiment 19. Points show the mean caries score of the control group (five animals, ●), the group immunized with SDS-washed walls prepared from a culture of *S. mutans* Ingbritt grown at $D = 0.5 \text{ h}^{-1}$ (four animals, ○) and the group immunized with SDS-washed walls from a culture grown at $D = 0.05 \text{ h}^{-1}$ (three animals, □).

Fig. 4. Levels of serum IgG antibody in monkeys of experiment 19 to antigens A, B, C and D determined by ELISA. The bars indicate the mean values obtained from 1, controls; 2, monkeys immunized with walls from culture grown at $D = 0.5 \text{ h}^{-1}$; 3, monkeys immunized with walls from culture grown at $D = 0.05 \text{ h}^{-1}$. Serum samples were from a test bleed 38.5 months after the start of the experiment and all were assayed in duplicate at a dilution of 1:200.

animals had significantly elevated levels of serum IgG antibody to antigens A, B and C, compared with control animals but the same method failed to demonstrate any significant difference in levels of antibody to antigen D.

Experiment 19 consisted of a group of control monkeys and groups immunized with walls from *S. mutans* strain Ingbritt grown at two different growth rates. Walls from the culture grown at a fast rate ($D = 0.5 \text{ h}^{-1}$) failed to confer any protection. No lesions developed in the primary dentition of the animals immunized with walls from the slow-grown culture ($D = 0.05 \text{ h}^{-1}$) and after 3 years on a high-sugar diet only a single lesion had developed in a permanent tooth of one animal (Fig. 3). Dental examinations of the non-protected immunized group were discontinued after 3 years, but monitoring of the remaining animals has confirmed that protection was still substantial after the monkeys had been on the high-sugar diet for a total of 45 months.

Serum antibody responses in monkeys of experiment 19 were assayed by ELISA using purified antigens A, B, C, D and also glucosyltransferase and glucan-binding protein. Immunization had no effect on levels of antibody to the last two. Data from a test bleed taken 2 weeks after the final booster injection (Fig. 4) showed that in all immunized animals there were

Fig. 5. The *Streptococcus mutans* populations in the plaque flora of monkeys in experiment 19, 25 months after introduction of the CHS diet, expressed as a percentage of the total streptococcus count. Redrawn from Table 1 of Beighton & Hayday, 1982. Group 1, controls; group 2, monkeys immunized with walls from culture grown at $D = 0.5 \text{ h}^{-1}$; group 3, monkeys immunized with walls from culture grown at $D = 0.05 \text{ h}^{-1}$. □, Serotype c; ▨, serotype h; ▩, serotype e; ■, serotype d/g. Each column represents a different animal.

raised levels of antibody to antigens A and B, though no antibody to antigen D could be detected and only the slow-growth rate walls had induced a significant response to antigen C. As had been observed previously (Russell & Beighton, 1982) control animals also develop natural antibody to *S. mutans* antigens. Marked differences were apparent in the levels of antibody to different antigens in the two immunized groups: the animals immunized with slow-growth rate walls (which were protected against caries) had higher levels of antibody to antigens A and C than did unprotected animals. The reverse was true of antibodies to antigen B, which were higher in the non-protected group. This difference in response between the two immunized groups was confirmed by ELISA of serum samples taken on several other occasions during the experiment, and also by titrating antibody to an end-point, a method more suited to detection low levels of antibody (Russell & Colman, 1981).

Bacteriology of group 19

The animals in experiment 19 have been the subject of a bacteriological study which determined the levels of different *S. mutans* serotype in individual monkeys (Beighton & Hayday, 1982). The relevant data are represented in Fig. 5.

Immunization with pure antigen D

Experiment 23 contained a group of six control monkeys, six immunized with antigen D and four immunized with a mutanolysin digest of *S. mutans* Ingbritt walls. The wall extract conferred protection against caries, albeit not as great as that observed with intact walls; after 34 months on the CHS diet the wall-immunized group had a mean of 1.25 caries lesions in their permanent teeth, with two of the animals still being caries-free. The non-immunized control group all had caries, the mean score being 3.3 while the scores of the group immunized with antigen D were not statistically significantly different from those of the controls, the mean being 3.4. The only animals that had developed an immune response to antigen D were those injected with the pure antigen.

Animals in experiment 23 were housed in communal cages, with the result that compared with animals housed individually much greater uniformity of bacterial flora was observed (Beighton & Hayday, 1982). Serotype *e* comprised approximately 60% of the streptococcal flora in the communes, serotype *c* 20% with only low numbers of serotypes *d*, *g* and *h* being detected.

DISCUSSION

Earlier reports from this laboratory described the characterization and purification of the protein antigens A and B (Russell, 1979*a, b*, 1980, 1981). These two antigens exist in forms which remain associated with walls even after being treated with SDS at 100 °C, a procedure believed to disrupt all except covalent bonds, although they are also released into culture supernatants. In the present report a milder procedure involving extraction of walls with SDS at room temperature was used but this still removed all membrane and cytoplasmic material; no further protein was removed by introducing more than two washes in SDS. This procedure allowed the recognition of the new antigen C but it is not clear whether antigen C is removed from walls by SDS at 100 °C or whether its antigenicity is destroyed. Although antigen D was not recognized by the methods used in earlier studies, it is apparent that it remains on walls even after treatment with SDS at 100 °C because walls so treated could induce an immune response to the antigen.

Antigens A and B were earlier postulated to be involved in the induction of protection against caries because monkeys immunized with walls had elevated levels of antibody to both antigens (Cohen *et al.*, 1979; Colman & Cohen, 1979). However, Cohen *et al.* (1979) also noted that protected animals had developed antibody to an antigen in addition to A and B and in the present paper we have shown this to be antigen C. No further antigens which might be involved in protection could be detected by crossed immunoelectrophoresis of either culture supernatant or mutanolysin-digested walls against antiserum to SDS-washed walls, nor did affinity chromatography on columns constructed with antiserum to walls reveal the existence of any further antigens. Our attempts to use the sensitive 'Western Blotting' technique to determine whether immune animals have antibodies to additional antigens have been frustrated by the problems arising from degradation of antigen B, the fragments of which show up as multiple bands on the nitrocellulose when treated with immune serum. Similar difficulties have attended the use of immunoprecipitation of radiolabelled antigens. The available evidence however, indicates that there are no further major protein components of the *S. mutans* wall prepared by our procedure, other than A, B, C and D.

Other workers have isolated antigens, named I, II and III, from *S. mutans* serotype *c* (Russell, 1979; Russell, *et al.*, 1980). The published values for molecular weights and isoelectric points of these antigens indicate that none of them are identical to antigens C or D, although antigens I and II can exist in a combined form (antigen I/II) which appears to correspond to our antigen B.

The results illustrated in Fig. 3 confirm the preliminary report by Cohen *et al.* (1979) that walls prepared from *S. mutans* grown at a slow-rate in a chemostat can induce effective protection against caries. Experiment 19 was designed to reproduce experiment C, started some 8 years earlier. In both experiments the same immunization schedule of multiple sub-mucosal injections without adjuvant was used. This regime was originally instituted in attempts to stimulate a local immune response but results already presented (Cohen *et al.*, 1979; Russell *et al.*, 1982) and those for experiment 23 in this paper have demonstrated that subcutaneous immunization at a site remote from the mouth is also effective. Such a route would probably be more acceptable in humans.

The monkeys in our colony harbour predominantly serotypes *c*, *e* and *h* of *S. mutans* and it has been shown that within a group of monkeys individuals may harbour different serotypes or combinations of serotypes (Beighton & Hayday, 1982). Immunization with antigens purified from serotype *c* should give protection against both serotypes *c* and *e* (and also *f*) because protein antigens of these serotypes are indistinguishable; serotypes carrying cross-reacting but non-identical antigens might also be protected against. Serotypes of *S. mutans* not carrying the antigen used for immunization should be unaffected. It is obvious that spurious results could be obtained from an experiment if different groups of animals carried different *S. mutans* serotypes, particularly if the colonizing strains lacked the antigen used as vaccine, but only recently has sufficient information become available to allow adequate bacteriological characterization of the monkeys' oral flora. In experiment 19, serotype *c* was the sole or predominant serotype of *S. mutans* in all animals except one, which carried serotype *e*. Since the wall antigens A, B and C are identical in serotypes *c* and *e*, the difference in caries experience between the groups cannot be attributed to differences in challenge by *S. mutans*. Similarly, in

experiment 23 for which the animals were housed in communal cages, all the animals carried serotype *e* on their teeth, the mean level being 60% of the total cultivable streptococci, while serotype *c* made up another 20%.

Comparison of the immune responses of monkeys in experiment 19, where the group immunized with one wall preparation was protected and the other not (Fig. 3), showed that the protected animals had the highest level of antibody to antigens A and C (Fig. 4). In contrast, the response to antigen B showed no correlation with protection. We are currently developing procedures for measuring amounts of the different antigens in wall preparations and our initial results (unpublished) have shown that *S. mutans* when growing at $D = 0.05 \text{ h}^{-1}$ produces substantially more antigen A than when growing at $D = 0.5 \text{ h}^{-1}$. Production of extracellular antigen B (referred to as protein P1) at different growth rates has already been measured by Hardy *et al.* (1982), who showed that more was produced at higher growth rates. Wall-bound antigen B (assayed by rocket immunoelectrophoresis of mutanolysin-digests) also increases with growth rate. The immune responses thus show a positive correlation with the levels of antigens A and B in the walls used as immunogens. Antigen C, however, does not appear to vary so markedly with growth rate so the variation in immunogenicity may be attributable to some other factor such as its conformation or location within the wall.

Antigen D, in its wall-bound form, appears to be a poor immunogen as only three of eight rabbits, and none of the monkeys, immunized with walls responded to it. It is clear that antigen D could not have been responsible for the protection of the wall-immunized monkeys and the experiment where it was used as a pure vaccine (which induced a strong specific response) confirmed its inability to protect.

Although the results from experiment 19 provide indirect evidence that antigens A and C alone or in combination could be responsible for the observed reduction in caries, the only way to test the importance of each antigen conclusively is to use each as pure single-component vaccines. Antigen C is currently under test; results on use of pure A and B have already been reported by Russell *et al.* (1982). Antigen A induced a high degree of protection (approaching 90% reduction in caries) in three separate groups of monkeys. Antigen B gave significant reduction in one experiment but this was not confirmed by a second. Apart from this lack of consistency of protection by antigen B, it should also be noted that antigen B appears to share antigenic determinants with heart tissue (Hughes *et al.*, 1980; Russell, 1979) and the possible hazard of inducing auto-immunity due to antibody to such a cross-reactive antigen means that B would be unacceptable for use as a human vaccine. Antigens A and C have not been shown to possess such heart cross-reactivity (M. Hughes, personal communication) and so hold considerable promise for possible use in humans.

We are grateful to David Beighton and Hazel Hayday for providing us with their data on the *S. mutans* population of the monkeys.

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