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IMMUNOGENICITY, BIOCHEMICAL AND SEROLOGICAL CHARACTERIZATIONS OF RIBOSOMAL PREPARATIONS FROM HUMAN ORAL STRAINS OF SEROTYPES *c* AND *d* OF THE BACTERIUM *STREPTOCOCCUS MUTANS*

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Summary—Crude ribosomal preparations of *Streptococcus mutans* C67-1 (serotype *c*) and 50B4 (serotype *d*) contain protein RNA and carbohydrate. Sepharose CL-2B column chromatography of preparations yielded two distinct peaks. Cell-wall carbohydrates were predominantly present in peak I; the serological activity resided mainly in peak II. The preparations contained antigens which cross-reacted with several streptococcal Lancefield antisera. Antisera prepared against the preparations cross-reacted with cell-wall proteins (NaCl extracts) and Ag I/II, but not with cell-wall carbohydrate antigens (Rantz–Randall extracts). Thus, cell-envelope protein antigens in the preparations appear to be responsible for the serological activity. The unique properties of ribosomal preparations may, apart from serological cross-reactivity, be useful in the immunological protection against dental caries.

INTRODUCTION

Streptococcus mutans is implicated as a principal microbial agent in dental caries in man (de Stoppelaar, van Houte and Backer Dirks, 1969; Huis in 't Veld *et al.*, 1979) and in experimental animals (Keyes, 1968). Effective protection against dental caries has been achieved in rats by immunization with whole cells of *Strep. mutans* (McGhee *et al.*, 1975) or cell-wall components (Taubman and Smith, 1976). Protection was associated with elevated serum IgG or salivary IgA antibodies to cell-wall antigens. Immunization of monkeys with whole cells of *Strep. mutans* also induced protection against dental caries and this was related to IgG class of antibodies and cell-mediated responses (Lehner *et al.*, 1976). Russell and Lehner (1978), Zanders and Lehner (1981) and Russell (1979) isolated a protein fraction from culture supernatants of *Strep. mutans* serotype *c* which, after one or two subcutaneous injections, provided long-term protection against dental caries in macaque monkeys (Lehner *et al.*, 1981; Russell, Beighton and Cohen, 1982).

Although *Strep. mutans* serotype *c* appears to be the predominant serotype in man (Bratthall, 1972; Perch, Kjems and Radin, 1974; Huis in 't Veld *et al.*, 1979) other *Strep. mutans* serotypes, as well as other streptococcal species, may contribute to the caries process. Ribosomal vaccines have been successfully used to protect rodents against otherwise lethal infections (Youmans and Youmans, 1965; Johnson, 1972; Schalla and Johnson, 1975; Gonggrijp *et al.*, 1980; Au and Eisenstein, 1981). Protective immune responses have been reported using ribosomal vaccines (Schalla and Johnson, 1975; Green and Johnson, 1980). Gregory and Shechmeister (1982, 1983) isolated ribosomal preparations from *Strep. mutans*. Immunization with these preparations interfered with colonization of the homologous strain in rats (Bozzola, 1981).

MATERIALS AND METHODS

Strains of Strep. mutans

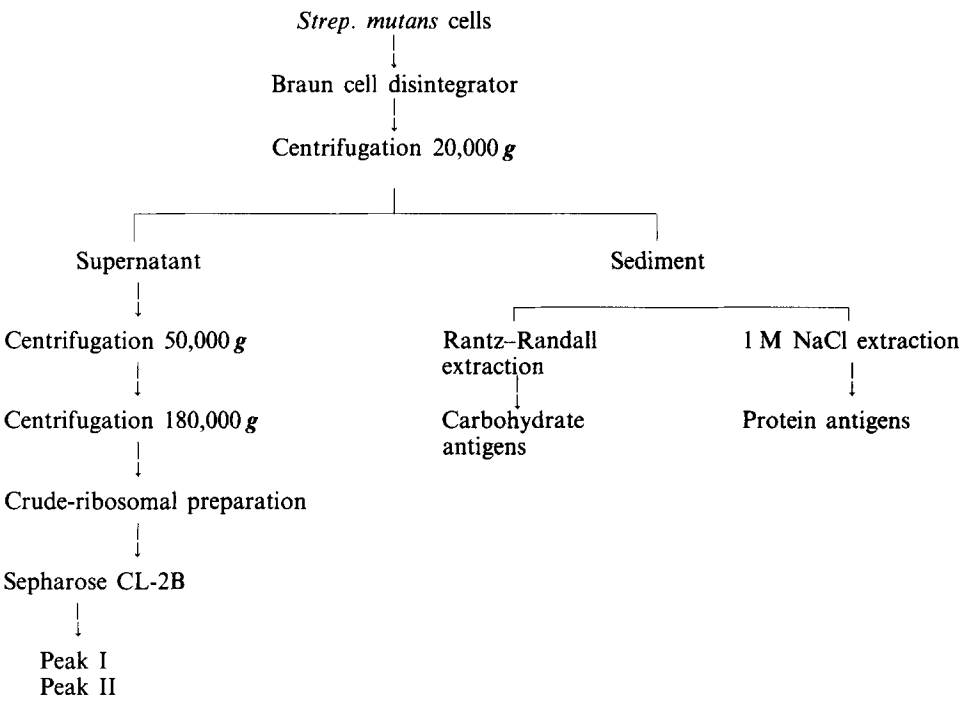
Strep. mutans C67-1 (serotype *c*) was originally isolated by de Stoppelaar *et al.* (1969). *Strep. mutans* 50B4 (serotype *d*) was isolated from dental plaque of a caries-active person (Huis in 't Veld *et al.*, 1979).

Extraction of antigens from Strep. mutans *cells* (*Scheme* 1)

Strep. mutans strain C67-1 and 50B4 were grown in 12 l of pre-warmed Todd–Hewitt Broth (Difco), containing 1 per cent glucose and 0.5 per cent $NaHCO_3$. The bacteria were collected by centrifugation and washed three times with 0.01 M tris-(hydroxy methyl) aminomethane (TRIS) buffer pH 7.2, containing 0.01 M KCl and 0.015 M $MgCl_2$ (TMK-buffer; Scheme 1). Washed cells were suspended in TMK-buffer (0.5 g wet wt/ml) and broken in a Braun-type cell disintegrator (B. Braun Co., Melsungen, Germany) for 5 consecutive 1-min periods at 4°C. Deoxyribonuclease (Boehringer, Germany) 0.2 μg/ml was added and the suspension incubated for 30 min at 37°C. The unbroken cells and cell debris were removed by centrifugation for 20 min at 20,000 ***g***. The pellets and whole *Strep. mutans* cells were used as a source of cell envelope antigens.

The supernatant was centrifuged for 30 min at 50,000 ***g***. Four-fifths of the supernatant was carefully removed and filtered through a 0.2 μm sartorius filter. The filtrate was centrifuged for 2.5 h at 180,000 ***g*** and the resulting pellet containing the ribosomes was suspended in TMK-buffer at a concentration of approx. 100 mg (wet wt/ml; crude ribosomal preparation). For chemical analyses, the preparations were dialysed and subsequently lyophilized.

Cell-wall protein Ag I/II was kindly provided by Dr T. Lehner, Guy's Hospital, London. Ribosomal preparations of *Strep. mutans* GS-5 were obtained



Scheme 1. Antigens preparation

from Dr J. Bozzola, Department of Microbiology, Medical College of Pennsylvania, U.S.A.

Molecular-sieve chromatography

Sepharose Cl-2B (Pharmacia, Uppsala, Sweden) was equilibrated at 4°C with TMK-buffer. The crude ribosome preparation (6–12 ml) was applied to a Sepharose C1-2B column (50 × 3 cm), eluted with the same buffer and the absorbance at 280 nm recorded. Two peaks were obtained. Fractions of each peak were combined, centrifuged for 2.5 h at 180,000 *g* and filtered through a 0.2 μm sartorius filter. The fractions were dialysed and subsequently lyophilized. Sterility of all vaccines was ensured by inoculating tubes (brain–heart infusion) and blood-agar plates with a drop of vaccine.

Extraction of cell-envelope antigens

Sedimented unbroken cells and cell walls were used as source for cell-wall carbohydrate and protein antigens. Carbohydrates were isolated by Rantz–Randall (RR) extraction (Rantz and Randall, 1955). Cell-bound proteins were isolated by 3 consecutive extractions with 4 ml 1 M NaCl. The washes were dialysed for 18 h, lyophilized and dissolved in 0.05 M phosphate buffer (pH 6.8).

Chemical analyses

The amount of RNA in the ribosomal fractions was determined by the method of Ogur and Rosen (1962). Total carbohydrates and protein were measured by the Anthrone (Radin, Lavin and Brown, 1955) and modified Lowry method respectively (Hartree, 1972). Qualitative identification of the carbohydrates was performed after acid hydrolysis of the ribosomal preparations (5 mg dry wt) in sealed tubes containing 1 ml 2 M HCl for 3 h at 100°C. The hydrolysates were neutralized with Dowex CO_3^{2-} and the samples were run on cellulose-FDC plates (Merck, Germany), using the solvent system butanol–pyridine–water (6:4:3). Carbohydrates were detected with the alkaline silver-nitrate reagent (Trevelyan, Proctor and Harrison, 1950).

Preparation of antisera

Mice. Immunogenicity of *Strep. mutans* C67-1 ribosomal preparations, cell-wall proteins and whole cells were compared in Balb/c mice. To this end, 28-day-old mice (12 per group) received, at 4-day intervals, 3 consecutive intraperitoneal injections of the antigens. Ribosomal and protein vaccines contained 50 μg material, in a total volume of 0.25 ml saline. Whole-cell vaccine was prepared by suspending *Strep. mutans* C67-1 in sterile saline, to an optical density of 4.8 at 540 nm. Control mice were sham-immunized with sterile saline. Blood was taken from the orbital plexus under ether anaesthesia.

Rabbits. Antisera to *Strep. mutans* C67-1 and 50B4 were prepared in rabbits by a course of 10 intravenous (i.v.) injections (0.5–2 ml) of u.v.-killed *Strep. mutans* cells (O.D. $\approx$ 6). Crude ribosomal preparations were suspended in TMK-buffer (30 mg/ml) and emulsified in an equal volume of Freund's complete adjuvant. Two rabbits were injected subcutaneously with 0.5 ml vaccine in the armpit and groin of the right fore and left hind-leg, followed by an intraperitoneal injection (0.5 ml) at day 15. When required, subsequent i.v. injections were given every fourth day. The animals were not bled until a strong capillary precipitation reaction had developed. Bleed-

ing took place 8 or 10 days after the last injection. Antisera to streptococcal Lancefield groups and *Strep. mutans* serotypes were kindly provided by Dr Groothuis, National Institute of Public Health, Bilthoven, The Netherlands.

Serological methods

Serum antibodies against *Strep. mutans* antigens were detected by either cell agglutination or the enzyme-linked immunosorbent assay (Elisa). In order to obtain cells which did not auto-agglutinate, Carlsson growth medium was used (Carlsson, Newbrun and Krasse, 1969). Cells were killed in 0.5 per cent formalin. Two-fold dilutions of the sera were made in phosphate-buffered saline (PBS) pH 6.8, containing 0.1 per cent bovine serum albumin and 0.037 per cent sodium ethylene diaminetetraacetate (EDTA). The cells were suspended in 0.1 per cent bovine serum albumin containing saline to an optical density of 0.45 at 540 nm and added to each serum dilution. The agglutination pattern was read after overnight incubation at 4°C. Elisa was carried out as described previously (Huis in 't Veld *et al.*, 1979). Antimouse IgG (Capel) was labelled with peroxidase by the method of Nakane and Kawoi (1974). Antibody titres were expressed as $\log_2$ of the last reciprocal dilution which showed a positive reaction.

Immunodiffusion was performed in 1 per cent (W/V) agarose in 0.05 M tris–HCl buffer pH 7.5 on glass slides. Slides were dried, washed in saline and stained with coomassie brilliant blue as described by Axelsen, Krøll and Weeke (1973).

Gel electrophoresis

Polyacrylamide gel electrophoresis (7.5 per cent; 0.5 × 8 cm) was carried out at 17°C, at 4.5 mA per tube, for 6 h (Knowles and Penefsky, 1972).

RESULTS

Chemical analyses of ribosomal preparations

The crude ribosomal preparation from *Strep. mutans* C67-1 and 50B4 contained significant amount of protein, RNA and carbohydrate (Table 1). The ratio of RNA/protein was 0.70 and 1.25, respectively.

Thin-layer chromatography (TLC) after acid hydrolysis yielded small amounts (<1 per cent) of typical cell-wall carbohydrates such as rhamnose, glucose and galactose. When crude ribosomal preparations were further purified on Sepharose CL-2B, two distinct peaks were obtained (Fig. 1). Protein and carbohydrate were the main components of peak I; peak II contained mainly protein and RNA (ratio about 1:1). Typical cell-envelope carbohydrates were present predominantly in peak I. The ratio of the dry weight of peaks I and II was 3:1 for *Strep. mutans* C67-1 and 1:1 for *Strep. mutans* 50B4. Polyacrylamide-gel electrophoresis (PAGE) of the crude ribosomal preparations from *Strep. mutans* C67-1 and 50B4 yielded 4 and 5 major bands respectively, 3 of which have identical positions (Fig. 2).

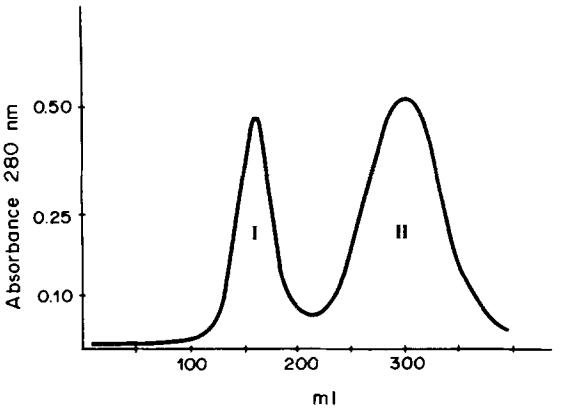


Fig. 1. Molecular-sieve chromatography of crude ribosomal preparations on Sepharose CL-2B column (50 × 3 cm). Amounts (6–12 ml) of the crude ribosomal preparation containing 180–360 mg (dry wt) was applied to the column and eluted with TMK buffer.

Immunogenicity of ribosomal preparations

Anti-*Strep. mutans* whole-cell serum showed in a cell-agglutination test, a $\log_2$ titre of 12 ± 0.6 (Fig. 3). In contrast, anti-ribosomal serum and antiserum raised against the cell-wall proteins showed titres which were only slightly higher than control serum. In the Elisa test, both sera gave a weak reaction with whole *Strep. mutans* C67-1 cells. When these sera were assayed against their homologous preparations, titres of up to $\log_2$ 10–11 were measured. Antiserum raised against cell-wall proteins strongly cross-reacted with the ribosomal preparation and vice versa, suggesting that proteins present in both fractions were responsible for the cross-reactivity.

Table 1. Chemical composition of crude ribosomal preparations and fractions I and II obtained after column chromatography on Sepharose CL-2B

		Protein*	RNA*	Carbohydrate*	Rhamnose†	Ribose†	Glucose†	Galactose†	RNA/prot
C67-1	Crude preparation	382	268	247	+	+	+ +	−	0.70
	Fraction I	321	52	116	+	+/−	+ +	−	0.16
	Fraction II	402	403	148	−	+ +	+ +	−	1.00
50B4	Crude preparation	384	480	101	+	+	+	+	1.25
	Fraction I	561	93	93	+	+/−	+ +	+	0.16
	Fraction II	429	484	67	−	+ +	+	−	1.13

*μg/mg dry weight. †Identification with thin-layer chromatography.

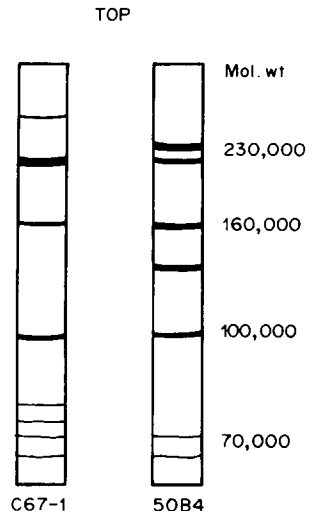


Fig. 2. Polyacrylamide gel electrophoresis of crude ribosomal preparations of *Strep. mutans* C67-1 (serotype *c*) and 50B4 (serotype *d*).

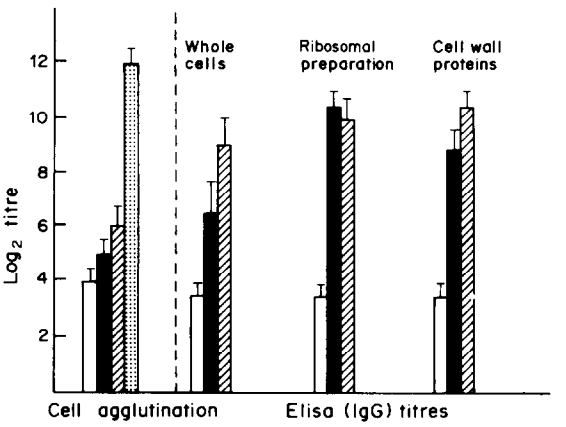


Fig. 3. Cell agglutination and Elisa titres (log_2 titre) of whole cells, ribosomal preparations and cell-wall proteins from *Strep. mutans* C67-1. Antisera tested: open column, control (negative) mouse serum; dark column, anti-ribosomal *c* serum; hatched column, anti-cell-wall protein serum; dotted column, anti-whole-cell serum.

Immunochemical analyses

The crude ribosomal preparations of both *Strep. mutans* strains showed cross-reactions with antisera against several streptococcal Lancefield groups in the capillary precipitation test (Table 2). The Rantz–Randall extracts of both strains precipitated only with the homologous antisera (results not shown). In immunodiffusion experiments, antiserum against whole cells of *Strep. mutans* C67-1 (*c*-serum) precipitated with crude ribosomes of both C67-1 (ribo *c*) and 50B4 (ribo *d*; Fig. 4a). The patterns suggested that a partial common antigen, present in ribo *c* and *d*, reacted with *c*-serum. The *c*-serum precipitated with the carbohydrate containing Rantz–Randall extract (RRc), the protein containing NaCl extracts (CW prot *c*) and with Ag I/II.

The antiserum against whole cells of *Strep. mutans* 50B4 (*d*-serum) precipitated only with ribo *d* and not with ribo *c* (Fig. 4b). Furthermore, *d*-serum precipitated with RRd and CW prot *d*. No precipitation was observed with Ag I/II. The pattern suggested that ribo *d* and RRd contained one common antigen. RRd and CW prot *d* showed precipitation lines which

Table 2. Serological reactions of crude ribosomal antigens with Streptococcal Lancefield sera

	Antigens Precipitin reaction	
Serum Lancefield group	C67-1	50B4
A	+ + +	+ + +
B	–	–
C	–	–
D	+	+ +
E	–	–
F	–	–
L	–	–
M	–	–
N	–	–
O	–	–
P	–	–
Strep. MG	+ +	+ +
Strep. mutans		
a	–	–
b	–	–
c	+ + +	+ +
d	+ / –	+ +
e	+	+ / –

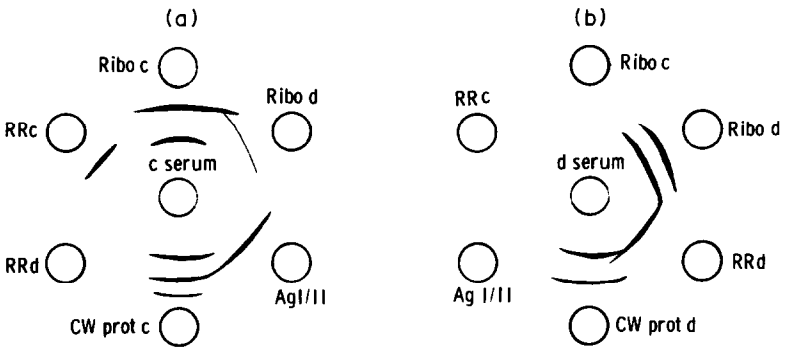


Fig. 4. Immunodiffusion of rabbit antiserum raised against whole cells of *Strep. mutans* C67-1 (*c*-serum) and 50B4 (*d*-serum) and their respective crude ribosomal preparations or cell-envelope antigens.

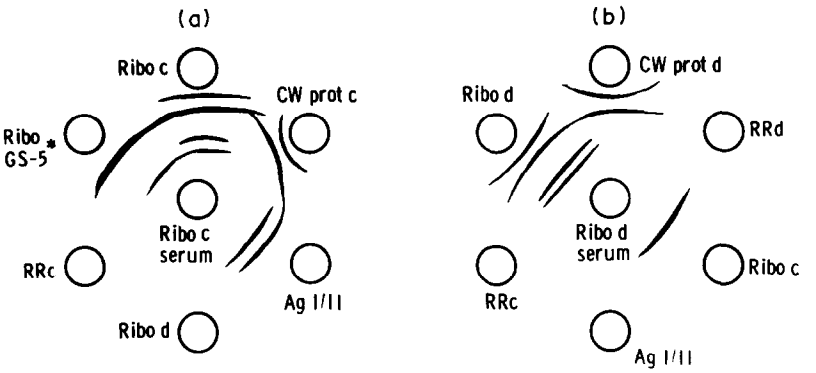


Fig. 5. Immunodiffusion of antiserum raised against crude ribosomal preparations of *Strep. mutans* C67-1 (ribo-*c*-serum) and 50B4 (ribo-*d*-serum) and crude ribosomal preparations or cell-envelope antigens.

suggest partial identity between antigenic compounds.

Antiserum against crude ribosomes of C67-1 (ribo-*c*-serum) precipitated against ribo *c*, CW prot *c*, Ag I/II and against a ribosomal preparation from *Strep. mutans* GS-5 (serotype *c*) which was obtained from Dr J. Bozzola (Fig. 5a). No precipitation was obtained with ribo *d* or with RRc. The results suggest the presence of two common antigens in ribo GS-5 and ribo *c*. A line of partial identity was observed between ribo *c* and CW prot *c*. CW prot *c* and Ag I/II also showed a line of identity against antiribo-*c*-serum.

Antiserum against crude ribosomes of *Strep. mutans* 50B4 (ribo-*d*-serum) precipitated with ribo *d*, CW prot *d* and ribo *c* (Fig. 5b). No precipitation was obtained against RRd and Ag I/II. In contrast to peak I, peak II of *Strep. mutans* C67-1 gave strong precipitation reactions with ribo-*c*-serum (Fig. 6). A line of partial identity was observed between peak II antigens and Ag I/II. As for C67-1, the major serological activity of *Strep. mutans* 50B4 also resides in peak II (data not shown).

DISCUSSION

Our findings characterized immunochemically the ribosomal preparations of *Strep. mutans c* and *d*.

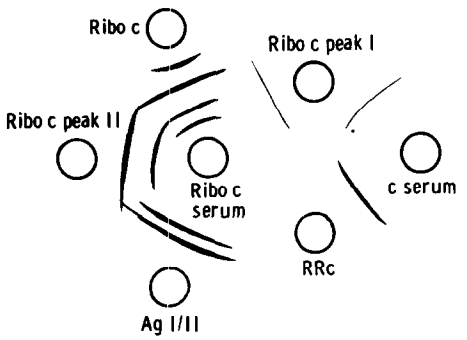


Fig. 6. Immunodiffusion of antiserum raised against whole cells or crude ribosomal preparations of *Step. mutans* C67-1 and crude ribosomal preparations, purified column fractions (peak I and peak II) of crude ribosomal preparations of C67-1 and cell-envelope antigens.

Although our data are in agreement with those found by Green and Johnson (1980) for *Strep. pyogenes* ribosomes (protein:RNA ratio 1:1), Gregory and Shechmeister (1983) found a different protein:RNA ratio (1:1.6) for *Strep. mutans* 6715. Methodological differences could be responsible for this, because ribosomes isolated from mechanically-disrupted cells appeared to possess greater protein: RNA ratios than ribosomes from enzymically-disrupted cells (Green and Johnson, 1980). SDS treatments, as used by Gregory and Shechmeister (1982), would certainly also influence the protein contents of ribosomal preparations. It is not known whether these proteins are of ribosomal origin or whether they are attached to the ribosomes during purification. Our results support, at least partly, the hypothesis that they become attached. Cell-wall proteins, such as Ag I/II and proteins which are extractable with 1 M NaCl showed immunodiffusion lines of partial identity with ribosomal preparations (Fig. 5). In contrast to Gregory and Shechmeister (1983), we found carbohydrates in both crude and peak-I ribosomal-like preparations. These findings have been confirmed by Misfeldt and Johnson (1977, 1978), Gonggrijp *et al.* (1981) and Green and Johnson (1980). Sensitive techniques are required to identify these carbohydrates amongst an excess of protein and RNA. The presence of lipoteichoic acids in the ribosomal preparation of Gregory and Shechmeister is, however, an indication of cell-envelope antigens in their preparations.

The crude ribosomal preparations from both serotypes of *Strep. mutans* contain antigens which cross-react with several Lancefield sera, especially with group *A*, *D* and *Streptococcus MG* anti-serum (Table 2). The Lancefield classification is based upon group-specific cell-wall carbohydrates, but antisera used for Lancefield classification also contain antibodies against other cell-wall components. Our evidence suggests that soluble as well as cell-associated proteins exist in certain streptococcal species which are common to several other oral bacteria (Bratthall and Pettersson, 1976; Schöller, Klein and Frank, 1981; Schöller *et al.*, 1982). It is therefore likely that some of these protein antigens were present in our ribosomal preparations. Indeed, in contrast to RNAse treatment, pronase treatment changes the immu-

noprecipitation pattern of ribosomal preparations (data not shown). Crude ribosomal preparations presumably contained sufficient cell-envelope antigens to precipitate with antisera raised against whole *Strep. mutans* cells (Fig. 4). Furthermore, experiments with antiserum raised against crude ribosomes showed only precipitations with protein containing extracts derived from whole cells, or cell walls, but not with carbohydrate-containing RR extracts (Fig. 5). Two of the components present in the ribosomal preparation of *Strep. mutans* GS-5 (serotype *c*; Fig. 4) also showed precipitation lines of total identity with antigens from C67-1 (serotype *c*).

Bacterial-surface antigens play an important role in host–parasite interactions. Several of those antigens such as glucosyltransferases and proteins have been used successfully to immunize animals against dental caries. In this respect, antigen I/II (Russell and Lehner, 1978; Zanders and Lehner, 1981) and protein *A* and *B* (Russell, 1979) have received considerable attention because of their caries-protective capacity in macaque monkeys. Although, Russell (1980) reported serological cross-reactivity between protein *A* and *B* and other streptococcal sera, little is known about the possible caries-protective effects of these antigens in animal experiments when heterologous bacteria are used in immunization. Ribosomal vaccines do not only confer protection *in vivo* against challenge with the homologous strains but also against heterologous serotypes.

Gregory and Shechmeister (1982, 1983) showed that *Strep. mutans* 6715 ribosomes induced both a humoral and a cellular response against all seven *Strep. mutans* serotypes. They also showed that antiserum raised against a ribosomal preparation was able to agglutinate formalin-killed cells of all seven representative serotypes of *Strep. mutans*. In addition, the *in vitro* adherence of *Strep. mutans* 6715 to glass surfaces was inhibited by antiribosomal sera. We have been unable to confirm these findings, because antisera raised against crude ribosomal preparations did not contain high levels of agglutinating antibodies (Fig. 3).

Differences in cell-agglutination titres were observed between anti-whole-cell serum and antiserum against ribosomal preparations and cell-wall proteins (Fig. 3.). As we ran out of anti-whole cell serum, we were unable to confirm these results in Elisa experiments. However, similar experiments carried out later with anti-whole cell *d* serum and whole *Strep. mutans d* cells showed the same pattern as in cell agglutination (Huis in 't Veld, data not shown).

The serological cross-reaction of our preparation with Ag I/II, together with the cross-protective effect of ribosomal preparation, justifies further research using purified subfractions of crude ribosomal preparations in studies involving the immunological protection against dental caries.

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