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A radioimmunoassay for serum and gingival crevicular fluid antibodies to a purified protein of *Streptococcus mutans*

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SUMMARY

A solid-phase radioimmunoassay was developed to measure serum IgG antibodies to a purified protein antigen I/II prepared from *Streptococcus mutans*. The assay was specific to this antigen and significant binding of ^{125}I-radiolabelled antiserum was found only in sera from rhesus monkeys immunized with the antigen I/II but not in sham-immunized monkeys or those immunized with streptococcal antigen III. A very significant correlation was found in serum IgG antibodies tested by the radioimmunoassay and an immunofluorescent technique ($r = 0{\cdot}88$, $P < 0{\cdot}001$). The sensitivity of the double-layer radioimmunoassay was increased 10 times by the addition of a third antibody layer and this enabled gingival crevicular fluid antibodies to be measured. Comparison of paired samples of serum and crevicular fluid revealed a very significant correlation between IgG antibodies to streptococcal antigen I/II in the two fluids ($P < 0{\cdot}001$). These findings suggest that serum antibodies can reach the tooth surface via gingival crevicular fluid.

INTRODUCTION

The isolation of specific protein antigens from *Streptococcus mutans* (Russell & Lehner, 1978; Russell *et al.*, 1980) and their protective effect in immunization against dental caries (Lehner, Russell & Caldwell, 1980) has led to a need for quantitative antibody assays, specific for the immunogen and the antibody isotype. With the demonstration that serum immunoglobulins can pass from the blood circulation into the gingival crevicular fluid (Challacombe *et al.*, 1978), a further requirement of the assay is high sensitivity which would allow antibodies in the gingival crevicular fluid to be assayed. A variety of tests have been used in the past to assess the antibody titre to the whole cells or cell wall components of *S. mutans*. Agglutinating assays were used with the cells of *S. mutans* (Taubman & Smith, 1974; McGhee *et al.*, 1975) and haemagglutinating, complement-fixing and precipitating antibodies with cell wall preparations (Lehner, Wilton & Ward, 1970; Lehner, Challacombe & Caldwell, 1976a). The indirect immunofluorescent test has been used to assay isotype-specific antibodies to surface components of the cells of *S. mutans* (Lehner *et al.*, 1976b). Antibodies were then examined against defined antigens prepared from *S. mutans*. Glucosyl transferase enzyme-inhibiting antibodies have been assayed in rats (Hayashi, Shklair & Bahn, 1972), rabbits (Evans & Genco 1973), man (Challacombe, Guggenheim & Lehner, 1973) and monkeys (Russell, Challacombe & Lehner, 1976). Haemagglutinating and complement-fixing antibody tests were used with serotype *c* polysaccharide, lipoteichoic acid and insoluble dextran derived from *S. mutans* (Russell, Challacombe & Lehner, 1980).

Antibody titres to the isolated protein antigens I/II, II and III from *S. mutans* were first tested by

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the precipitation technique (Russell *et al.*, 1980). Antibody-forming cells to dinitrophenylated streptococcal antigen I/II were assayed in investigations of both T cell helper and suppressor activities (Lamb, Kontiainen & Lehner, 1979, 1980). Furthermore, opsonizing antibodies to the streptococcal antigens I/II, II and III have also been detected in phagocytosis with neutrophils (Scully, Russell & Lehner, 1980).

The aims of this investigation were to develop a solid-phase radioimmunoassay using the streptococcal antigen I/II. This was based on the technique described by Catt & Tregear (1967) and modified by Hay, Nineham & Roitt (1975).

MATERIALS AND METHODS

Antisera. The IgG fraction of rabbit anti-rhesus monkey IgG (Fc) (Nordic Immunological Laboratories, Berkshire) was purified by passage through DEAE-cellulose in 0·0175 M phosphate, pH 6·5. The IgG fraction of swine anti-rabbit immunoglobulin was obtained from DAKO Immunoglobulins AS (Copenhagen, Denmark).

Antigens. Streptococcus mutans antigen I/II (SAI/II) was prepared from the culture supernatant by the method of Russell & Lehner (1978) and Russell *et al.* (1980); antigen III (SAIII) was prepared by the method of Russell (1979).

Sera. These were collected from 25 monkeys immunized with SAI/II (12 monkeys), whole cells (three monkeys), SAIII (two monkeys) and from those sham-immunized with saline (eight monkeys). Samples were collected before and then 4, 8, 32 and 100 weeks after immunization. For standardization of the assays, reference immune and control samples were included in each experiment; sera from six monkeys immunized with SAI/II were pooled for the immune sample, as were the sera from two monkeys injected with saline for the control sample. All sera were divided into 100-μl aliquots and stored at either -20°C or, in the case of the reference sera, at -70°C.

Crevicular fluid washings (CFW). These were collected from the same monkeys by the method of Skapsky & Lehner (1976), centrifuged, and stored at -20°C.

Protein estimations. Concentrations of protein in the IgG preparations were estimated spectrophotometrically by absorption at 280 nm. SAI/II and SAIII protein concentrations were determined by the method of Lowry *et al.* (1951), using bovine serum albumin (BSA; Sigma) as a standard.

Radioiodination. The IgG fraction of the antiserum was radiolabelled with ^{125}I (IMS-30, The Radiochemical Centre, Amersham) by modification of the method of Hunter & Greenwood (1962). One milligram of antibody in 0·2 ml phosphate-buffered saline (PBS) and 10 μl of 0·5 M phosphate, pH 7·5, was incubated with 1 mCi ^{125}I and 10 μl chloramine T (5 mg/ml) for 1 min at room temperature. The reaction was stopped by the addition of 10 μl sodium metabisulphite (12 mg/ml) and 150 mg sodium iodide. Radiolabelled protein was separated from free iodine by passage through Sephadex G-50, diluted to 10 ml in 0·5% BSA in PBS, with 0·02% sodium azide and stored at 4°C. This bound approximately 80% of the ^{125}I to the protein.

Double-antibody radioimmunoassay. Polystyrene tubes (LP3; Luckham Ltd, Sussex) were coated with 0·75 ml of SAI/II at a concentration of 1 μg protein per ml in PBS and 0·02% sodium azide for 20 hr at 20°C. Unbound antigen was decanted and the tubes were incubated with 2·5 ml of 0·5% BSA in PBS, containing 0·05% polyoxyethylene sorbitan monolaurate (Tween 20), for 1 hr at 37°C to prevent further non-specific interactions. The tubes were washed three times with PBS–Tween 20 and then incubated in duplicate with 0·5 ml monkey serum, diluted in BSA–PBS–Tween 20 for 3 hr at 37°C. After three washes the tubes were incubated with 0·75 ml ^{125}I-radiolabelled rabbit anti-monkey IgG (Fc) at a concentration of 1 μg/ml in BSA–PBS–Tween 20 for 2 hr at 37°C. The tubes were washed three times and bound ^{125}I-radiolabel was assessed in a Beckman gamma counter. Controls included in each experiment were coated tubes (a) without serum and (b) the reference immune and control sera. Results were expressed as a percentage of the reference immune serum assayed at a dilution of 1:50.

Triple-antibody radioimmunoassay. The tubes were coated and blocked as in the double-antibody assay. They were then washed three times and incubated in duplicate with 0·5 ml of monkey

serum or CFW, diluted in BSA–PBS–Tween 20, for 2 hr at 37°C. The tubes were washed and then incubated with 0·75 ml rabbit anti-monkey IgG (Fc) at a concentration of 10 μg/ml in BSA–PBS–Tween 20 for 1 hr at 37°C. After washing three times, the tubes were incubated with 0·75 ml of ^{125}I-radiolabelled swine anti-rabbit IgG at a concentration of 1 μg/ml in BSA–PBS–Tween 20 for 1 hr at 37°C. The tubes were then washed three times and bound ^{125}I-radiolabel was assessed as in the double-antibody assay. Controls included in each experiment were coated tubes (a) without serum or CFW but with rabbit anti-monkey IgG (Fc), (b) with serum or CFW but without rabbit anti-monkey IgG (Fc), (c) without serum, CFW and rabbit anti-monkey IgG (Fc) and (d) the reference immune and control sera.

Determination of optimal concentrations of SAI/II and antisera in the two assays. Concentrations of SAI/II from 0·00064 to 10 μg/ml were used in the assays with the reference immune and control sera at a dilution of 1:50. Using tubes coated with SAI/II at a concentration of 1 μg/ml and the reference immune and control sera at a dilution of 1:50, the radiolabelled rabbit antiserum was titrated from 0·0016 to 5 μg/ml in the double-antibody assay. In the triple-antibody assay tubes coated with 1 μg/ml SAI/II were incubated with the reference immune and control sera at dilutions of 1:50, 1:5,000, 1:500,000 and without any serum. The rabbit anti-monkey IgG (Fc) was added at concentrations of 0, 1, 5 and 10 μg/ml and the ^{125}I-radiolabelled swine anti-rabbit immunoglobulin at concentrations of 1, 5 and 10 μg/ml.

Sensitivity of the assays. The reference immune and control sera were titrated in both the double- and triple-antibody assays from a dilution of 1:50 to 1:5,000,000.

Specificity of the assays. This was tested by competitive inhibition of the sera. One millilitre of a polyspecific monkey serum at a dilution of 1:2,500 was incubated overnight at 4°C with 5 μg of SAI/II, SAIII, or BSA–PBS–Tween 20 alone. The serum was centrifuged and specific antibody binding was measured in the double-antibody assay using SAI/II- and SAIII-coated tubes.

Reproducibility of the assays. The reference immune and control sera were titrated on 11 different occasions in the double-antibody assay and on six different occasions in the triple-antibody assay.

Titration of sera and CFW. The sequential sera collected from immunized and control monkeys were titrated from 1:50 to 1:50,000 in the double-antibody assay. CFW taken at about 100 weeks after immunization from the same monkeys were assayed at a dilution of 1:10 in the triple-antibody assay.

Immunofluorescent test. The indirect method was used against air-dried smears of *S. mutans*, (Lehner *et al.*, 1976b). Monkey sera were serially diluted from 1:5 and a rabbit anti-human IgG (Fc) fluorescein conjugate (Wellcome Laboratories) was used to visualize bound antibodies.

RESULTS

Standardization of the assays. Bound ^{125}I-radiolabel was detectable at the lowest concentration of SAI/II used. This increased with the concentrations of SAI/II, reaching a plateau at 1 μg/ml SAI/II (Fig. 1). Above 2 μg/ml the amount of ^{125}I-radiolabel bound in the presence of immune serum was reduced in the double-antibody assay, probably due to an unstable complex of multiple layers of antigen on the tube. A concentration of 1 μg/ml SAI/II was used in all subsequent assays. The optimum concentration of ^{125}I-radiolabelled rabbit antiserum in the double-antibody assay was found to be 1 μg/ml. Optimal conditions in the triple-antibody assay were achieved with the use of 1 μg/ml SAI/II, 10 μg/ml rabbit anti-monkey IgG (Fc) and 1 μg/ml of ^{125}I-radiolabelled swine anti-rabbit immunoglobulins.

Titration of immune and control sera. Titration of the reference sera in the double- and triple-antibody assays is shown in Fig. 2. The curves for the immune serum in both assays are sigmoid, with comparable binding of ^{125}I-radiolabel at dilutions of 1:50 and 1:500. Specific antibody in the immune serum was detectable at a dilution of 1:50,000 on titration in the double assay and an equivalent binding of ^{125}I-radiolabel was obtained at a serum dilution of 1:500,000 in the triple assay. At these dilutions the binding of the control serum was within the background level of the assays. These results suggest that the triple assay is at least 10 times more sensitive than the double assay.

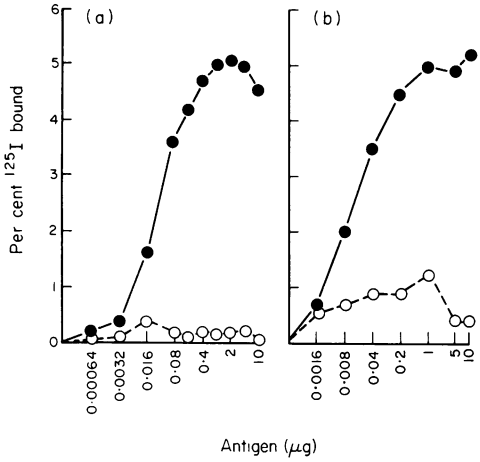

Fig. 1. Titration of SAI/II in (a) the double- and (b) the triple-antibody radioimmunoassays. Binding of immune serum (●——●) and control serum (o- - -o) at a dilution of 1:50 is expressed as a percentage of the total ^{125}I-radiolabelled antiserum.

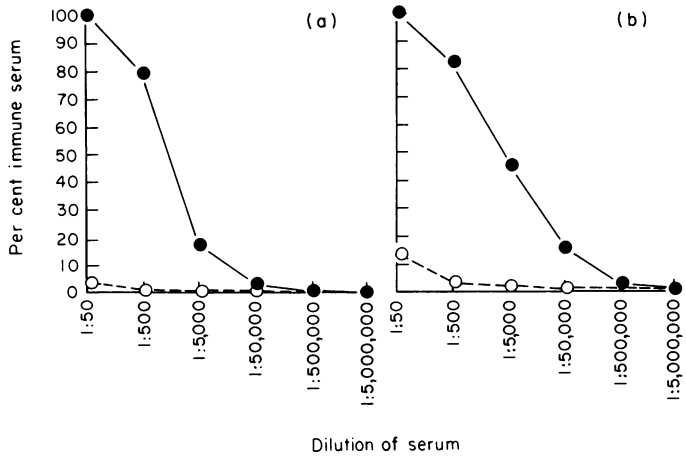

Fig. 2. Titration of immune serum (●——●) and control serum (o- - -o) against SAI/II in (a) the double-antibody and (b) the triple-antibody assay. Results are expressed as a percentage of the immune serum binding at a dilution of 1:50.

Table 1. Solid-phase radioimmunoassay for serum IgG antibodies to streptococcal antigen I/II in five groups of monkeys

Group	No. of animals	Serum IgG mean (±s.e.)*
Preimmunized	20	1·9 (0·6)
Sham-immunized	6	3·0 (1·2)
SAIII	2	8·4
SAI/II	11	73·4 (6·3)
Whole cells	3	94 (4)

* Results expressed as a percentage of the immune serum binding at a dilution of 1:50. The test sera were diluted at 1:500.

SA = streptococcal antigen.

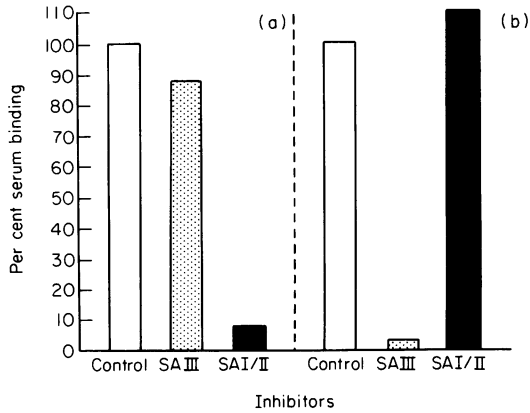

Fig. 3. Competitive inhibition of binding of a polyspecific serum to (a) SAI/II-coated tubes and (b) SAIII-coated tubes by free SAI/II and SAIII at 5 μg/ml. Results are expressed as a percentage of the serum binding at a dilution of 1:2,500.

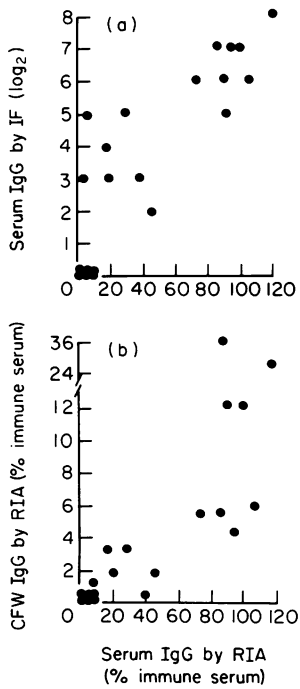

Fig. 4. Correlation between (a) serum IgG antibodies to SAI/II detected by radioimmunoassay (RIA) and cell surface antigens detected by immunofluorescence (IF) and (b) serum and crevicular fluid washing (CFW) IgG antibodies to SAI/II by RIA.

Specificity of the double-antibody assay. SAI/II inhibited the binding of the polyspecific monkey serum to SAI/II-coated tubes by 92% but made little difference (10% enhancement) to the binding of the same serum to SAIII-coated tubes (Fig. 3). Conversely, SAIII reduced the binding of the same serum to SAIII-coated tubes by 97% with little reduction (10%) of the binding of the serum to SAI/II-coated tubes. Thus, the assay is specific for the antigen under investigation.

Reproducibility. For immune serum dilutions giving 50% binding, the reproducibilities of the assays showed a coefficient of variation of 7% for the double-antibody assay at a serum dilution of 1:1,000 and 13% for the triple-antibody assay at a serum dilution of 1:5,000.

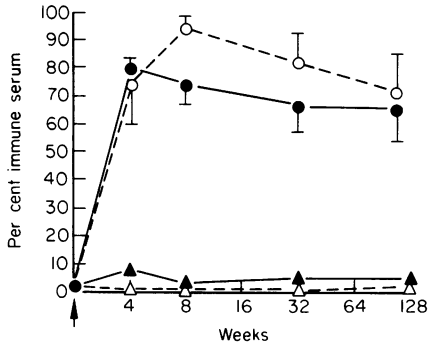

Fig. 5. Sequential levels of IgG antibodies to SAI/II in monkeys immunized with SAI/II (●——●), whole cells (o- - -o), SAIII (▲——▲) and controls (△- - -△) at a dilution of 1:500. Results are expressed as a mean (±s.e.) percentage of the reference immune serum.

Table 2. Solid-phase radioimmunoassay for IgG antibodies to streptococcal antigen I/II in paired serum and CFW from three groups of monkeys

Group	No. of animals	Serum IgG mean (±s.e.)*	CFW IgG mean (±s.e.)*
Sham-immunized	8	6·6 (4·6)	0·3 (0·1)
SAIII	2	4·8	0·5
SAI/II	12	72·2 (10·1)	10 (3·2)

* Results expressed as a percentage of the immune serum binding at a dilution of 1:50. The test sera were diluted at 1:500 and CFW at 1:10.

SA = streptococcal antigen, CFW = crevicular fluid washing.

Serum IgG antibodies. Significant IgG antibody titres to SAI/II were found by the double-antibody assay in those monkeys immunized with SAI/II or whole cells, whilst those immunized with SAIII or injected with saline yielded negligible binding (Table 1). A very significant correlation was established between the serum IgG antibodies assessed by the radioimmunoassay and the immunofluorescent test (Fig. 4a; $r = 0{\cdot}8806$, $P < 0{\cdot}001$). Sequential analysis of the sera over a period of about 2 years showed that the high titres of antibodies elicited within 4 weeks of immunization with SAI/II or whole cells were well maintained. However, sera from monkeys immunized with SAIII or those injected with saline showed negligible binding (Fig. 5).

CFW IgG antibodies. As measured in the triple-antibody assay, the binding of ^{125}I-radiolabel was significantly higher in the CFW taken from SAI/II-immunized monkeys than in the CFW taken from SAIII-immunized and saline-injected monkeys (Table 2). A significant correlation was found between paired serum and CFW IgG antibodies to SAI/II using the Spearman rank correlation test (Fig. 4b; $r = 0{\cdot}8882$, $P < 0{\cdot}001$).

DISCUSSION

A solid-phase radioimmunoassay has been developed to quantitate IgG antibodies to SAI/II. The assay showed good reproducibility and was sensitive to an immune serum dilution greater than 1:50,000. The assay was specific to the protein antigen SAI/II, as antibody binding was inhibited by SAI/II but not by SAIII derived from the same organism (Fig. 3).

The serum IgG antibodies to SAI/II from monkeys immunized with SAI/II, SAIII or sham-immunized, as assessed by the radioimmunoassay, showed a very significant correlation with the

IgG fluorescent antibody test against cells of *S. mutans* ($P < 0{\cdot}001$). This is consistent with the finding that antigen I/II is expressed on the surface of cells of *S. mutans* (Russell & Lehner, 1978; Russell *et al.*, 1980), so that the fluorescent antibody test can be used as an indirect measure of antibodies to SAI/II. However, the serum which showed a titre greater than 1 : 50,000 by the double radioimmunoassay, reached a titre of only 1 : 2,560 by the immunofluorescent method. The sensitivity of the radioimmunoassay was increased 10 times by converting it from a double- to a triple-antibody assay. This enabled serum antibodies to be titrated to dilutions greater than 1 : 500,000 and could be used to detect specific antibodies in crevicular fluid. The small quantities and high dilutions of crevicular fluid (Challacombe *et al.*, 1978) collected by the gingival washing technique (Skapsky & Lehner, 1976) required the high sensitivity of the triple assay. Although avidity of the antibodies was not determined, the finding that antibodies can be assayed at a dilution of 1 : 500,000 argues in favour of high-avidity antibodies. By changing the isotype specificity of the second antibody, the triple assay may be used for the detection of specific IgA or IgM class of antibodies.

Comparison of paired samples of serum and crevicular fluid revealed a very significant correlation between IgG antibodies in the two fluids ($P < 0{\cdot}001$). This is not surprising as the antibodies in crevicular fluid originate from blood, though up to 20% can be produced by the local gingival plasma cells (Brandtzaeg, Fjellanger & Gjeruldsen 1970; Challacombe *et al.*, 1978). Hence, antibodies detected in serum are present in crevicular fluid and therefore at the tooth surface, where bacterial colonization takes place.

Antibodies of the IgG class appear to be associated with protection against dental caries in rhesus monkeys (Lehner *et al.*, 1976b, 1979) and in man (Challacombe & Lehner, 1976; Lehner *et al.*, 1978a). Furthermore, passive transfer of IgG from monkeys immunized with *S. mutans* induced protection from dental caries (Lehner *et al.*, 1978b). Antibodies in crevicular fluid may function by preventing adherence of *S. mutans* to the tooth or may opsonize the bacteria for phagocytosis and killing by polymorphonuclear leucocytes (Scully & Lehner, 1979). A further point arising from the significant correlation between serum and crevicular fluid IgG class of specific antibodies is the possible use of CFW instead of serum in assaying serum components particularly in children.

The radioimmunoassay for the detection of IgG class of antibodies to SAI/II uses the purified antigen at relatively low concentrations. The assay is specific and more sensitive than other methods used for the detection of antibodies to purified antigens of *S. mutans* and should be a suitable method in assessing the antibodies in serum or gingival crevicular fluid in man.

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