Immunology 1984 **52** 143

Natural antibodies in man to *Streptococcus mutans*: specificity and quantification

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Accepted for publication 25 November 1983

Summary. Antibodies to whole cells of *Streptococcus mutans* were examined in 108 subjects by a solid-phase radioimmunoassay and quantified by reference to isotype-specific affinity-purified antibodies. Serum antibodies of each isotype were present in all subjects examined. The mean concentration of serum antibodies to *S. mutans* was calculated as about 84 μg/ml of IgG (range 33–140 μg/ml), 26 μg/ml of IgA (range 12–43 μg/ml) and 9 μg/ml of IgM (range 4–15 μg/ml). The mean antibody values accounted for about 0·7, 1 and 0·8% of the total IgG, IgA, and IgM, respectively. Overall the antibody binding to whole cells of *S. mutans* accounted for about 0·8% of the total immunoglobulin. Inhibition experiments using a variety of purified cell wall antigens revealed that the binding of antibodies to whole cells could be inhibited by about 30% with a purified protein antigen (SA I/II) and with glucosyltransferase (GTF), by 25% with c polysaccharide and by 16% with lipoteichoic acid. The protein antigens GTF and SA I/II appear to be major immunogenic cell wall antigens, but natural antibodies in man that bind to *S. mutans* whole cells have been induced by several antigens, some of which are specific to *S. mutans* and some of which are shared with other Gram-positive bacteria.

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INTRODUCTION

Natural antibodies have been detected in human serum to a wide variety of bacteria and are frequently used to help in the diagnosis of disease. In recent years serum antibodies to oral bacteria have been examined by many authors with the aims either of determining any role of serum antibodies in the oral cavity or of relating specific bacteria to a particular oral disease. (For reviews see McGhee & Michalek, 1981; Tolo & Brandtzaeg, 1982.)

There has been particular interest in the relationship between antibodies to *Streptococcus mutans* and dental caries, since *S. mutans* is thought to play an important part in the aetiology of dental caries in man. It is present in small numbers in the plaque of most people, but the numbers are markedly increased if dental caries is present (see McGhee & Michalek, 1981). Seven different serotypes (a–g) have now been identified (Bratthall, 1970; Perch, Kjems & Raun, 1974) on the basis of carbohydrate antigens in the cell wall, though the predominant serotype in man appears to be serotype c (Hamada, Masuda & Kotani, 1980; Keene *et al.*, 1977).

Natural antibodies to *S. mutans* have been detected in serum (Lehner, Wilton & Ward, 1979; Berkenbilt & Bahn, 1971; Challacombe, Guggenheim & Lehner, 1973; Challacombe, 1980; Orstavik & Brandtzaeg, 1977; Huis int'Veld van Palenstein Helderman & Backer Dirks, 1979) and in saliva (Challacombe & Lehner, 1976; Arnold, Mestecky & McGhee, 1976; Bratthall *et al.*, 1979). Serum IgG antibody titres

against *S. mutans* are significantly greater in subjects of low caries experience than subjects of high caries experience when subjects with carious lesions are excluded (Challacombe, 1980; Challacombe & Lehner, 1976). The presence of carious lesions leads to a raised antibody titre (Challacombe, 1980). In these studies specificity to *S. mutans* was indicated because antibodies to several other oral cariogenic organisms including *S. sanguis*, *Actinomyces viscosus*, *S. salivarius* and *Lactobacillus acidophilus* did not show any relationship with caries (Challacombe & Lehner, 1976).

The antigens of *S. mutans* to which these natural antibodies are directed have not been identified, but it has been suggested that antibodies to lipoteichoic acid (LTA) may be responsible for a large part of the antibody activity (Bratthall *et al.*, 1979; Russell R.R.B. & Beighton, 1982). However, LTA is only one of a number of antigens found in the cell wall of *S. mutans*, which include glucosyltransferases (Guggenhiem, 1970), serotype-specific carbohydrates (Mukasa & Slade, 1973), and various protein antigens (Russell & Lehner, 1978).

The objectives of this investigation were to examine the specificity of natural antibodies in man reacting with whole cells of *S. mutans*, and to attempt to quantify the amount of antibody of each class reactive with *S. mutans*.

MATERIALS AND METHODS

Subjects

The series consisted of 108 healthy young adults whose ages ranged from 18 to 25 years, with a mean of 21·1 years. The index of decayed, missing or filled teeth (DMF) ranged from 0 to 24 with a mean of 10·8. Thirty subjects had one or more carious lesions. All subjects had clinically normal gingiva with a gingival index of less than 0·4 (Loe & Silness, 1963). Samples of serum were taken and treated as previously described (Challacombe & Lehner, 1976).

Bacteria

Streptococcus mutans OMZ 61 (serotype a), FAl (b), Guy's (c), OMZ 176 (d), LM7 (e), OMZ 175 (f), 6715 (g), *S. sanguis* OMZ 9, *A. viscosus* (WVU 371) and *L. casei* were grown, harvested, and stored as described elsewhere (Czerkinsky *et al.*, 1983).

Antigens from S. mutans

Cell walls were prepared by ultrasonication and differential centrifugation (Challacombe, 1974). Antigen I/II (SA I/II) is a cell wall-associated protein antigen with a molecular weight of 185,000 (Russell & Lehner, 1978) and was extracted from culture supernatants as previously detailed (Russell *et al.*, 1980). The protein gave a single band on sodium dodecyl sulphate (SDS) gels (Zanders & Lehner, 1981).

Other antigens were prepared essentially as described previously (Russell, Challacombe & Lehner, 1980b). Lipoteichoic acid (LTA) was prepared from a serotype c strain of *S. mutans* by the aqueous phenol method (Wicken, Gibbens & Knox, 1973). After phenol extraction, the aqueous phase was recovered by centrifugation, and after dialysis was digested with ribonuclease, deoxyribonuclease and pronase. LTA was than purified by gel filtration on Sepharose 6B (Pharmacia Ltd), and was found to be immunochemically pure.

Serotype c-specific polysaccharide was extracted from whole cells using 5% trichloroacetic acid (Linzer, Gill & Slade, 1976) and purified by DEAE cellulose chromatography.

Insoluble dextran was isolated from serotype c cells by the method of McCabe & Smith (1975). The culture medium was supplemented with 2% sucrose. Bacterial cells were pelleted by centrifugation and treated with 20% KOH for 1 hr at 100°. The solution was adjusted to pH 8 with glacial acetic acid, and the precipitate was recovered and treated twice more with 20% KOH and acetic acid before being washed.

Glucosyltransferases were prepared from cells as described by Kuramitsu (1974). *S. mutans* were grown in a dialysate medium (Fukui, Fukui & Morujama, 1974) and about 40 g of cells extracted three times with 1 M NaCl at 4° for 16 hr. The pooled supernatants were centrifuged, concentrated by ultrafiltration, and after dialysis were lyophilized. Immunoelectrophoretic analysis revealed that the preparation contained trace amounts of SA I/II and LTA.

Immunoglobulin concentrations

Concentrations of IgG, IgA and IgM in serum were assayed by single radial immunodiffusion as previously described (Challacombe, 1976), using a laboratory pooled serum standardized against an international reference serum (WHO 67/99) and containing 1200 mg/100 ml of IgG, 240 mg/100 ml of IgA and 96 mg/100 ml of IgM.

Radioassay of antibodies

Serum antibodies to whole cells of *S. mutans* were assayed by a solid-phase radioimmunoassay (RIA) as described elsewhere (Czerkinsky *et al.*, 1983). Briefly, whole bacterial cells at a concentration of about 2×10^9/ml were bound to the solid phase with 0·3% methylglyoxal. After incubation with the samples of serum at a dilution of 1 in 200 or 1 in 1000, isotype specificity was achieved by incubation with the IgG fractions of rabbit anti-human IgG, IgA or IgM, (DAKO Ltd) followed by radiolabelled swine anti-rabbit (DAKO Ltd). The antisera were used at concentrations of about 4 μg/ml. The antibody binding of each sample was calculated in relation to a standard curve constructed from a pooled normal human serum assayed on the same occasion (see, for example, Fig. 1), and which contained a known

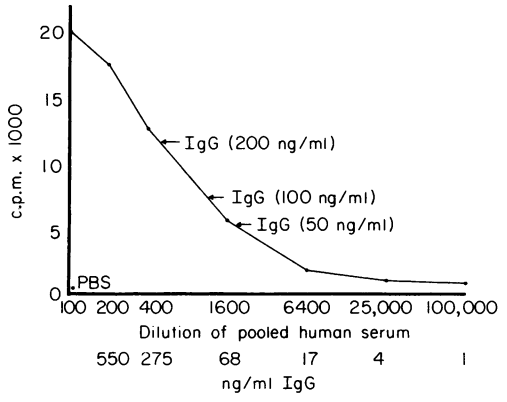

Figure 1. Standardization of a pooled normal human serum in ng/ml of IgG antibody to *S. mutans*. The serum was diluted from $1:10^2$ to $1:10^5$ and calibrated against known concentrations of purified specific antibody of the IgG isotype. The antibody content of serum samples was then read from the standard serum curve.

amount of antibody of each isotype. An assay dilution of 1 in 200 was selected since the antibody binding of the majority of samples fell on the linear portion of the standard curve. Some sera of high titre were assayed at 1 in 1000.

Affinity chromatography

Human sera which showed high antibody activity to *S. mutans* were pooled and then separated into IgG, IgA, and IgM fractions by DEAE cellulose chromatography (Challacombe, 1974). The IgG, IgA and IgM fractions were further purified by affinity chromatography on Sepharose 4B (Pharmacia) to which anti-human IgG, IgA or IgM had been coupled according to the manufacturer's instructions. Each purified fraction was then passed through an affinity column containing formalin-fixed cells of *S. mutans* attached to AH-sepharose (Pharmacia). Bound antibody was eluted with glycine-HCl buffer at pH 2·8, and dialysed against saline. The protein concentration of the purified IgG, IgA, and IgM anti-*S. mutans* antibody was assayed (Lowry *et al.*, 1951).

Calibration of pooled serum standard

The antibody content of the pooled human serum standard was calibrated against each of the preparations of purified isotype specific antibody in the indirect RIA (see, for example, Fig. 1), assuming that the c.p.m. using a given amount of purified antibody was equivalent to the same amount of antibody in the pooled serum. The pooled human serum was calculated to have 110 μg/ml of IgG antibody to *S. mutans* and 30 μg/ml of IgA antibody and 15 μg/ml of IgM antibody.

Iodination

Antisera were radiolabelled with ^{125}I (IMS-3, Amersham International) by a modification of the chloramine-T method as described elsewhere (Challacombe, Russell & Hawkes, 1978).

Specificity of antibodies

Sera which showed high antibody activity to *S. mutans* serotype c were absorbed with a variety of bacteria including other serotypes of *S. mutans* and other streptococci, and then re-examined for antibody to *S. mutans* serotype c in the RIA. Sera were absorbed with packed bacterial cells (at 1/8 of the volume of serum) for 1 hr at 37° followed by overnight incubation at 4°. Any anti-immunoglobulin serum which showed significant binding to *S. mutans* was also absorbed in this way.

In inhibition experiments, human sera at a dilution of 1:200 were inhibited with purified antigens from *S. mutans* at concentrations of between 1 and 100 μg/ml, and with cell walls at 1 mg/ml. Antigens were incubated with the serum samples for 1 hr at 37° before assay, and the subsequent binding of antibodies to whole cells compared with samples that had been incubated with saline.

RESULTS

Serum antibodies to *S. mutans*

The calculated concentrations of IgG, IgA and IgM antibodies in serum to whole cells of *S. mutans* are shown in Table 1. IgG antibody concentrations ranged from 33 μg/ml to 140 μg/ml with a mean concentration of 84·2 μg/ml. IgA antibody concentrations ranged from 12 μg/ml to 43 μg/ml with a mean of 26·0 μg/ml, and IgM antibody concentrations ranged from 4 μg/ml to 15 μg/ml with a mean of 9·3 μg/ml. Total antibody levels against *S. mutans* ranged from 63 to 195 μg/ml (Table 1).

The IgG antibodies accounted for about 70% of the total specific antibody, and represented about 0·7% of the total serum IgG immunoglobulin. IgA antibodies accounted for about 22% of the total antibody, and the mean value of 26 μg/ml represented 1% of the total serum IgA in these subjects. IgM antibodies were found at a mean concentration of 9·3 μg/ml (about 0·8% of the total serum IgM) which accounted for 8% of the antibody to *S. mutans* (Table 1). The mean immunoglobulin concentrations in serum were 12·02 mg/ml for IgG, 2·54 mg/ml for IgA and 1·12 mg/ml for IgM (Table 1).

Subjects with carious lesions had significantly greater amounts of specific antibody of the IgG ($P < 0·001$) and IgM ($P < 0·05$) classes than subjects without carious lesions (Table 2).

Specificity of antibodies to whole cells of *S. mutans*

Sera from six subjects showing high antibody titres to whole cells were diluted 1 in 200 for the inhibition experiments, and each assayed separately. Preincubation of the serum samples with cell walls of *S. mutans* resulted in about 60% inhibition of the antibody binding to whole cells. With purified antigens the greatest inhibition of antibody binding to whole cells was seen after preincubation with GTF, SA I/II, and with the serotype c-specific polysaccharide (Fig. 2). At a concentration of 10 μg/ml significant inhibition was found only with the c polysaccharide GTF and SA I/II. At a concentration of 100 μg/ml about 30%

Table 1. Proportion of serum antibody binding to *Streptococcus mutans*

	IgG	IgA	IgM	Total
Specific antibody in μg/ml (mean ± SD)	84·2 ± 29·0	26·0 ± 14·1	9·3 ± 3·8	119·3 ± 28·6
Range	33–140	12–43	4–15	63–195
Total immunoglobulin Conc. in μg/ml mean ± SD)	12,020 ± 2830	2540 ± 690	1120 ± 490	15,280 ± 2320
Antibody as % of total Ig	0·70 ± 0·29	1·02 ± 0·41	0·83 ± 0·35	0·78 ± 0·23
Number of subjects	108	75	75	75

Antibodies assayed against standard curve calibrated from affinity purified isotype specific antibodies (see 'Methods').

Table 2. Concentrations of serum antibody binding with *Streptococcus mutans* in relation to the presence of carious lesions (μg/ml ± SD)

Isotype	Subjects with lesions (30)	Subjects without lesions (78)	*t*	*P*
IgG	103·3 ± 37·4	72·6 ± 26	4·125	< 0·001
IgA	27·1 ± 15·8	25·4 ± 13·1	0·501	NS
IgM	10·9 ± 4·1	8·7 ± 3·7	2·056	< 0·05

Method: see Table 1.

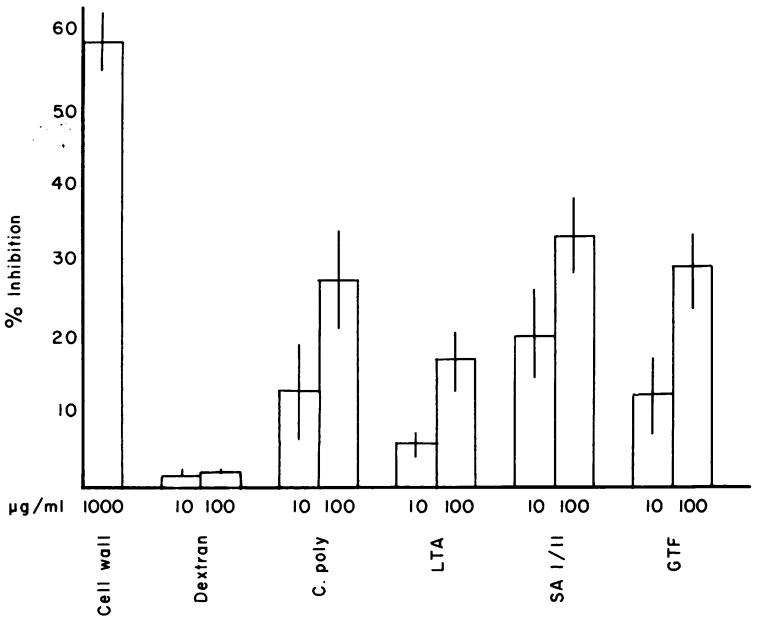

Figure 2. Inhibition of antibody binding to whole cells of *S. mutans* by purified streptococcal antigens. Inhibition by 10 or 100 µg/ml of antigens or by 1000 µg/ml of cell wall is compared with the value after treatment with saline (mean ± SD of six subjects).

inhibition was achieved with GTF, SA I/II and with the c polysaccharide, whereas about half this inhibition was found with LTA and virtually no inhibition was detected with dextran.

Absorption of the pooled normal human serum with different bacteria showed that the antibody binding to *S. mutans* serotype c could be reduced by 85% with serotype c organisms and by over 70% with the related e and f serotypes (Fig. 3). Other serotypes of *S. mutans* reduced the antibody binding by between 20 and 30%. Some reduction was found with all other organisms examined ranging from 9% with *A. viscosus* to 24% with *L. casei* (Fig. 3).

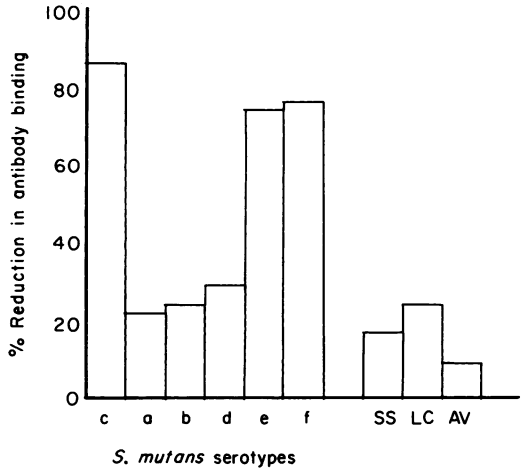

Figure 3. Binding of antibody to *S. mutans* serotype c whole cells after absorption of pooled normal human serum with different bacteria. SS = *Streptococcus sanguis*, LC = *Lactobacillus casei*, AV = *Actinomyces viscosus*.

DISCUSSION

In the present investigation antibodies to whole cells of *S. mutans* were assayed by a solid-phase radioimmunoassay. The use of methylglyoxal in the RIA binds bacteria firmly to the solid phase (Czerkinsky *et al.*, 1983), and with this assay quantification of antibodies is possible. In this study, the quantity of IgG, IgM and IgA antibodies in a pooled normal serum was calculated on the basis of calibration against affinity purified isotype specific anti-*S. mutans* antibodies. This is a method that has been used elsewhere for the calibration of antibodies (Stevens & Saxon, 1979; Yarchoan *et al.*, 1981). Quantification relies on the assumption that a given c.p.m. in the standard serum equates in quantity with the same c.p.m. of a known

amount of affinity-purified antibody. This may not necessarily be so because the affinity-purified antibody may be partly denatured during the purification process and the affinity or avidity of antibodies in a serum may differ from the pooled serum. Nevertheless the method enables a reasonable estimation of the quantity of antibody to be made.

The mean IgG antibody concentration to *S. mutans* in these subjects was about 84 μg/ml, compared with 26 μg/ml for IgA and 9 μg/ml for IgM (Table 1). Thus IgG accounted for over 70% of the total specific antibody binding to *S. mutans* compared with 79% of the total immunoglobulin. The proportions of IgM as a percentage of the total specific antibody, or as a percentage of the total immunoglobulin was similar at about 7·5%. There seemed to be a relative increase in the proportion of IgA specific antibodies since 22% of the total specific antibody to *S. mutans* was comprised of IgG compared with 17% of the total immunoglobulin. This finding is consistent with observations that raised serum IgA levels are found in association with oral and gastrointestinal diseases (Hobbs, 1969; Lehner, 1969).

The proportion of the total immunoglobulin in each class that was specific antibody was also calculated in each subject. A mean of 0·7% of the IgG, 0·8% of the IgM and 1·0% of the IgA was immunoglobulin which bound to *S. mutans*. This bacterium contains many antigens, some of which are specific to the species, while others are common to other streptococci or to other Gram-positive bacteria (Bratthall & Pettersson, 1976; Scholler, Klein & Frank, 1981). Some of the antibody binding with *S. mutans* may therefore have been induced by cross-reacting antigens elsewhere, and it would be expected that the proportion of immunoglobulin reacting with any defined antigen would be considerably less. In addition, any denaturation of the affinity-purified antibody would lead to an overestimation of the biologically active antibody in the standard serum, and thus to an overestimation of the values in the serum samples.

It is interesting to note that Stevens *et al.* (1979) reported that after a booster immunization with tetanus toxoid in five volunteers, the serum IgG antibody concentration increased to about 120 μg/ml from a resting level three- to 10-fold lower. This indicates that, after immunization, about 1% of the total IgG was specific antibody to tetanus toxoid, and prior to booster immunization 0·1–0·3% was specific antibody. The observation in the current study, that 0·8% of the total immunoglobulin of each class is antibody binding to *S. mutans*, is comparable, especially considering the multi-antigen nature of a bacterial cell. It also suggests that frequent immunization with *S. mutans* or cross-reacting bacteria may occur. Since small numbers of this organism are found in the plaque of most subjects, immunization could occur through contact with the gingiva, via bacteraemias or through carious lesions (Challacombe, 1976). It is interesting to note that, in this study, subjects with carious lesions had significantly greater amounts of IgG and IgM antibody than subjects without lesions. This can be interpreted as reflecting a serum response to *S. mutans* infection.

The specificity of natural antibodies to whole cells of *S. mutans* was examined in this study by antibody inhibition with purified antigens, and by absorption with other bacteria. The results suggest a high degree of specificity of antibodies, because absorption with a serotype c strain of *S. mutans* reduced the antibody titre to serotype c by greater than 85%, and the genetically related serotype e and f strains reduced the titre by over 70%. This was in contrast to absorption with unrelated Gram-positive bacteria *A. viscosus*, *L. casei* or *S. sanguis*, or with a, b and d serotypes of *S. mutans*, which reduced the antibody titres by a maximum of 30% (Fig. 3). This specificity is similar to that found with rabbit antisera to *S. mutans* (Czerkinsky *et al.*, 1983).

The cell wall of *S. mutans* contains a number of specific and shared antigens (Bratthall & Pettersson, 1976; Scholler *et al.*, 1981). The inhibition studies suggested that natural antibodies to *S. mutans* in man could be accounted for by several different antigens. Antibodies to LTA appeared to be only a minor proportion of the total antibody directed against whole cells of *S. mutans*, and serotype c polysaccharide, SA I/II and GTF were all capable of inhibiting natural antibodies to whole cells to a greater extent (Fig. 2). These findings are similar to inhibition experiments with rabbit antisera to azide-inactivated whole cells of *S. mutans*, where significant inhibition was found with SA I/II and GTF but not with LTA or dextran (Czerkinsky *et al.*, 1983). These findings do not substantiate the suggestion that antibodies to LTA might account for much of the antibody directed against *S. mutans* (Bratthall *et al.*, 1979; Russell R.R.B. & Beighton, 1982), and suggest rather that natural antibodies in man binding to whole cells of *S. mutans* appear to be a combination of antibodies to a number of cell wall antigens. Immunization of rhesus monkeys with *S. mutans* cells also leads to the

development of serum antibodies directed against several different cell wall antigens (Russell *et al.*, 1980b).

The results of this study suggest that in human sera about 0·8% of the total immunoglobulin in each antibody class will bind to whole cells of *S. mutans*. These natural antibodies appear to reflect the response to a number of different cell wall antigens, some of which are specific to *S. mutans*, others of which are shared with other bacteria.

ACKNOWLEDGMENTS

This study was supported by a grant from the Medical Research Council which is gratefully acknowledged.

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