Albumin-binding proteins on the surface of the *Streptococcus milleri* group and characterization of the albumin receptor of *Streptococcus intermedius* C5

M. D. P. WILLCOX,* M. PATRIKAKIS, C. Y. LOO and K. W. KNOX

Institute of Dental Research, United Dental Hospital, Sydney, NSW 2010, Australia

(Received 16 February 1993; revised 26 April 1993; accepted 5 May 1993)

Members of the *Streptococcus milleri* group (SMG) that react with Lancefield group C antisera were shown to bind large amounts of albumin although there was no direct relation between these two properties as polyclonal antisera to Lancefield group C antigen did not prevent the binding of albumin. There was a specificity for albumin binding, with albumin from man, monkeys, cat, dog and mouse being bound to a greater degree than albumin from cow, horse, goat or rabbit. Gold-labelled albumin was shown to be located close to the surface of strains by transmission electron microscopy. A cell-surface protein of M_r 24000, which was liberated by lysozyme treatment of cells, was shown to be the cell-surface receptor on *Streptococcus intermedius* C5. The receptor was physically dissimilar from protein G, an albumin- and IgG-binding protein of 'large-colony' Lancefield group C and G streptococci.

Introduction

The *Streptococcus milleri* group (SMG) is composed of three related species, *Streptococcus anginosus*, *Streptococcus intermedius* and *Streptococcus constellatus* (Whiley & Hardie, 1989). Members of all species have been isolated from the human oral cavity, which is considered to be their environmental niche (Mejr & Edwardsson, 1975), and where they have been implicated in dental caries (Drucker & Green, 1978) and dental abscesses (Williams *et al.*, 1983). Their occurrence at other body sites indicates that they may be commensal (Ruoff, 1988), though their isolation from severe suppurative infections in many organs in the human body, including the liver (Moore-Gillon *et al.*, 1981), the central nervous system and from patients with infective endocarditis (Gossling, 1988), indicates their pathogenic potential. To this extent it has been realized in recent

years that SMG strains may be 'unrecognized pathogens' (Ruoff, 1988). Most of the strains possess Lancefield group antigen A, C, F or G (Whiley *et al.*, 1990; Gossling, 1988) and several investigations have been made into the relation between the 'large-colony' Lancefield group A, C or G streptococci (particularly *Streptococcus equi* and *Streptococcus dysgalactiae*) and the 'minute-colony' SMG, especially as both colony types can be isolated from similar sites (Lawrence *et al.*, 1985; Cimolai *et al.*, 1988).

Information on the pathogenic determinants of the SMG is limited. However, certain strains do have the capacity to bind fibronectin, fibrinogen, albumin (Willcox & Knox, 1990; Willcox *et al.*, 1992), laminin (Switalski *et al.*, 1987), β_2 -microglobulin (Ericson *et al.*, 1980) and/or plasminogen (Ullberg *et al.*, 1989). Studies on the binding of albumin and IgG by large-colony streptococci of Lancefield groups C and G have demonstrated that the cell-surface molecule protein G is responsible for the binding of both proteins and also α_2 -macroglobulin by some strains (Lmmler *et al.*, 1988; Wideback *et al.*, 1982). The albumin receptors from the various species have been shown to bind specific albumins; for example, *S. dysgalactiae* strains (group C strains) show specificity for bovine serum albumin (Wideback & Kronvall, 1982; Raeder *et al.*, 1991). To date at least five different types of albumin receptor have

* Author for correspondence. Present address: Cooperative Research Centre for Eye Research and Technology, University of New South Wales, PO Box 1, Kensington, NSW 2031, Australia. Tel. 2 931 0213; fax, 2 931 0202.

Abbreviations: BHI, brain heart infusion; POW, Prince of Wales Hospital; SMG, *Streptococcus milleri* group; SLS, sodium lauroyl-sarcosinate; PBST, phosphate buffered saline containing Tween 20; LTA, lipoteichoic acid.

been described based on their ability to bind albumin from different animal species. It has also been proposed that lipoteichoic acid, a common amphiphile associated with the cell wall of most species of streptococci, can bind to albumin (Simpson *et al.*, 1980) and may account for the low-affinity binding levels seen with many streptococcal cells.

Analogies with the interaction of other streptococci with mammalian proteins point to possible pathogenic functions for albumin binding. The binding of albumin to group A streptococci increases phagocytosis but not intracellular killing (Wagner *et al.*, 1986). It has been demonstrated that there may be some protective advantage in abscess-producing bacteria being phagocytosed by abscess-derived neutrophils, which are inefficient at killing bacteria (Finlay-Jones *et al.*, 1991).

The aim of the present investigation was to examine strains within the SMG for their ability to bind albumin from several animal species, to localize the receptor for albumin by electron microscopy, to characterize the receptor on the surface of a strain and to determine whether the receptor was similar to protein G.

Methods

Bacterial strains and growth conditions. Table 1 shows the strains used and their site of isolation. The SMG were kindly supplied by Dr J. Tappsell, Prince of Wales Hospital, Sydney (POW strains) and Dr K. Ruoff, Massachusetts General Hospital, Boston, USA. Large-colony group C (*Streptococcus dysgalactiae*) and G strains were the kind donation of Professor L. Björck, University of Lund, Sweden, and

were included as positive controls for albumin binding and DNA-DNA hybridizations. *Staphylococcus aureus* strain STA3 was from our freeze-dried culture collection and was included in the study as a negative control in DNA-DNA dot-blot hybridizations. Strains were stored at -70°C in 30% (v/v) glycerol.

All strains were grown in brain-heart infusion (BHI; Oxoid) supplemented with 0.3% (w/v) yeast extract (Difco) at 37°C for 18 h in an anaerobic jar. Cultures for biochemical characterization of strains were grown on Columbia-blood agar (Oxoid), with 5% (v/v) sheep's blood added, for 18 h at 37°C in an anaerobic atmosphere, unless otherwise stated.

Biochemical characterization and Lancefield grouping. The biochemical characterization of SMG strains followed the scheme of Whiley *et al.* (1990), with some modification. All streptococcal strains were tested for fermentation reactions in API 20Strep. test kits (API) and for the production of enzymes in API ZYM test kits and using substrates labelled with the fluorescent marker 4-methylumbelliferyl (Beighton & Whiley, 1990). Production of hyaluronidase was determined by the rapid plate method of Smith & Willet (1968). Lancefield grouping was performed on whole, washed cells from an overnight broth culture using the Streptex latex agglutination kit (Wellcome Diagnostics).

Binding of albumin to strains. All albumins, with the exception of rat albumin, were obtained from a commercial source (Sigma) and are shown in Fig. 1. Rat albumin was prepared by passing fresh rat plasma through a blue-Sepharose affinity column (Pharmacia LKB) and eluting with an NaCl gradient in PBS ($\text{NaCl } 8 \text{ g l}^{-1}$, $\text{KCl } 0.2 \text{ g l}^{-1}$, $\text{Na}_2\text{HPO}_4 \text{ } 1.15 \text{ g l}^{-1}$, $\text{KH}_2\text{PO}_4 \text{ } 0.2 \text{ g l}^{-1}$). The purity of all albumins was confirmed by polyacrylamide gel electrophoresis and showed a single band at M_r 63000. Albumins were labelled with ^{125}I according to the method of Markwell (1982) and ^{125}I -albumin was separated from unbound ^{125}I by chromatography on PD-10 columns (Pharmacia LKB) containing 9.1 ml Sephadex G-25M, followed by dialysis against PBST [PBS containing Tween 20 (0.5% v/v)].

The method of Willcox & Knox (1990) was chosen to measure the binding of albumin to bacteria, with minor modifications. Cells from

Table 1. *Characteristics of Streptococcus strains used*

Strain no.	Species	Site of isolation	Lancefield group	Rat albumin bound (molecules per cell) $\pm$ SD†
POW1	<i>S. anginosus</i>	Appendiceal abscess	C	2768 ± 176
C117221-1	<i>S. anginosus</i>	Perirectal abscess	C	2553 ± 172
C5	<i>S. intermedius</i>	Throat swab	C	2828 ± 137
POW3	<i>S. intermedius</i>	Cerebral abscess	—*	141 ± 20
POW5	<i>S. constellatus</i>	Blood	F	35 ± 29
F4	<i>S. anginosus</i>	Sputum	F	155 ± 17
G14	<i>S. anginosus</i>	Sputum	G	101 ± 23
G23	<i>S. constellatus</i>	Sputum	G	818 ± 141
G148	Large-colony group G	Throat	G	1919 ± 70
G43	Large-colony group G	Throat	G	1764 ± 138
C40	Large-colony group C	Throat	C	1295 ± 150

* The strain was non-groupable with Lancefield antisera.

† 1×10^{-11} mol ^{125}I -labelled albumin added to 5×10^8 cells.

broth cultures were washed and resuspended to an OD_{660} of 1.0 (1×10^9 cells ml^{-1}) in PBST. The Tween 20 was added to reduce non-specific binding to cells. Bacterial suspension (0.5 ml) and 1×10^{-11} mol albumin were allowed to interact for 30 min at ambient temperature (22–26 °C). Bacteria were separated from unbound ^{125}I -albumin by centrifugation (5000 g, 10 min) and washed three times in PBST. The radioactivity associated with the bacterial pellet was then measured in a gamma counter. Control tubes containing no bacterial cells were also run and any radioactivity associated with these tubes was subtracted from the test tubes. The amount of binding was expressed as number of molecules per bacterial cell.

Initial experiments compared the ability of all bacteria to bind to rat albumin. Subsequently, strain C5 was tested for its ability to bind to other albumin types. Finally, other group C SMG, strains F4, G14 and G23 and large-colony group C and G strains were compared for their ability to bind to rat, human, rabbit and cow albumins in order to assess the conservation of the specific binding.

Localization of albumin receptor. Human albumin was coupled with colloidal gold by an established technique (Beesley, 1989) and allowed to interact with bacteria (strains C5, C117221-1, POW1 or F4) on a carbon-Formvar grid as described previously (Wyatt *et al.*, 1988) using PBST as the buffer. After negative staining with methylamine tungstate, the grids were examined by transmission electron microscopy.

Factors affecting albumin binding. Strain C5 was used exclusively for further study into the molecular nature of the receptor for albumin. To test for any effect of subculturing, cells were grown on Columbia-blood agar, passaged into BHI and then sequentially passaged on agar and into broth until the culture had been grown in broth 30 times. The cells were then assayed for their ability to bind to rat albumin. In other experiments, cells (1×10^9 ml^{-1}) were treated with either proteinase K (Sigma), lipase (Sigma) or periodate. For periodate treatment, cells were resuspended in 0.1 M-sodium phosphate buffer (pH 6.5) containing 20 mM-periodic acid, incubated for 20 min at 4 °C and washed in $NaBH_4$ (Sandgren *et al.*, 1991). After treatment, the cells were resuspended to an OD_{660} of 1.0 in PBST and allowed to interact with rat ^{125}I -albumin in solution.

The ability of substances to interfere with binding was measured by incubating cells in 1 mg ml^{-1} of either non-immune IgG, rabbit IgG to *S. mutans* protein P1 (Forester *et al.*, 1983), serum to group C polysaccharide (Difco), or rabbit IgG to lipoteichoic acid (Wicken & Knox, 1971), or in an equal concentration (1×10^{-11} mol) of human albumin, bovine albumin or ovalbumin (Sigma). The ability to affect the number of rat ^{125}I -albumin molecules was assessed either after treatment (IgG) or by adding the rat albumin together with the other albumins. Statistical analysis, using Spearman's rank test, was performed to demonstrate which treatments had an effect.

Extraction of receptor. Approximately 9 g wet weight of cells (from a 6-litre culture of strain C5) was divided equally for extractions by the nine procedures described below.

Cells were boiled for 10 min in either PBS (neutral extraction), in PBS adjusted to pH 2 (with HCl) or adjusted to pH 10 (with NaOH), or in PBS containing 0.5% sodium dodecyl sulphate (SDS extraction). The cells were then separated by centrifugation and the supernatants adjusted to pH 7.0. These methods are modifications of methods used previously to extract albumin receptors from 'large-colony' (*S. dysgalactiae*) group C streptococci (Raeder *et al.*, 1991).

For enzymic liberation of receptor, cells were incubated at 37 °C for 18 h with either 0.2 units insoluble trypsin (Sigma; Lämmle *et al.*, 1987), 5 mg lysozyme (Sigma; Lämmle & Sting, 1989), 10 mg mutanolysin (Sigma) in 10 ml PBS or 10 mg mutanolysin in 10 ml 0.02 M-Tris/HCl buffer (pH 7.0) containing 0.01 M-MgSO₄ and 40% (w/v) raffinose (this solution prevents cell lysis; data not presented). Cells were removed by centrifugation and filtration through

0.22 µm filters. Cell-surface proteins were also extracted with 1% (w/v) sodium lauroylsarcosinate (SLS) for 20 min at ambient temperature followed by centrifugation (Jenkinson, 1986). All extracts were stored at –20 °C until required.

To assess the extraction of a functional receptor and its molecular mass, molecules were separated by SDS-PAGE on 4–15% gels using a Phast system (Pharmacia LKB). Gels were either stained with Coomassie blue or subjected to Western blotting in a semi-dry transfer apparatus attached to the Phast system. After transfer to nitrocellulose membranes, the membranes were washed three times in PBST [containing 5% (w/v) skim milk powder] and then incubated with 200 µg ^{125}I -human albumin in PBST + milk for 1 h. After three washes in PBST, autoradiography was performed at –20 °C for 48 h.

Purification of receptor. An affinity column of human albumin Sepharose was prepared by treating CNBr-labelled Sepharose (Pharmacia LKB) with human albumin according to the manufacturer's instructions. The active extract (containing a functional receptor), in 50 mM-Tris/HCl (pH 7.5), was applied to the column and washed sequentially with 1 M-NaCl, 1 M-urea, 4 M-urea and 0.2 M-glycine/HCl buffer (pH 2.6). Fractions were then subjected to SDS-PAGE and autoradiography (as described above) to identify those containing the receptor.

Characterization of purified receptor. The purified receptor (20 µg) was allowed to interact with 1×10^{-11} mol ^{125}I -human albumin in solution for 30 min at ambient temperature before adding 5×10^8 cells of C5 in PBST for 30 min at ambient temperature. The cells were separated from solution by centrifugation (5000 g, 10 min) and washed three times in PBST. The purified receptor was also incubated with 20 mg (protein) insoluble trypsin (Sigma) for 1 h at 37 °C. After incubation, the insoluble trypsin was removed by centrifugation (5000 g, 10 min) and the supernatant fraction tested for its ability to interfere with albumin binding to whole cells. To test for the ability of the purified receptor to bind IgG, the purified receptor was subjected to SDS-PAGE, Western blotted onto nitrocellulose and then incubated for 1 h in PBST + milk (see above) containing ^{125}I -human IgG. Excess IgG was removed by washing in PBST + milk and the nitrocellulose was then subjected to autoradiography.

Dot-blot Southern hybridization. Chromosomal DNA was isolated from all the strains. After growth in BHI for 18 h at 37 °C, the cells were centrifuged, resuspended in 20 ml fresh BHI and incubated for 2 h at 37 °C. The cells were then washed once in TE buffer (50 mM-Tris/HCl pH 7.5; 20 mM-EDTA), mutanolysin (125 units) was added and the cells were incubated at 60 °C until a marked decrease in opalescence of the suspension was observed (usually between 1 and 2 h). After lysing cells by the addition of 20 µl 20% (w/v) SDS, 0.06 mg proteinase K was added and incubated at 37 °C for 1 h. Protein was then precipitated by the addition of 200 µl saturated NaCl solution and removed by centrifugation (7000 g, 10 min). DNA was then precipitated with 0.6 vols 2-propanol. After centrifugation and drying the pellet, the DNA was resuspended in TE, incubated with RNAase (final concn 10 mg ml^{-1}) to remove residual RNA and extracted with an equal volume of phenol, followed by phenol/chloroform and finally chloroform (Sambrook *et al.*, 1989).

An oligonucleotide probe (AAATATGGAGTAAGTGACTAT) that corresponded to a conserved portion of protein G gene that encodes for the albumin-binding region (Ottén & Boyle, 1991) was synthesized using a Gene Assembler Plus synthesizer (Pharmacia LKB). The probe was then labelled with ^{32}P using a commercially available kit (Amersham).

Chromosomal DNA was made single-stranded by boiling and then 20 ng dotted onto a nylon membrane (Hybond N+; Amersham). Using commercially available buffers and blocking agents (Amersham) the membrane was probed with 10 ng of the oligonucleotide probe at

42 °C. After washing with $0.5 \times$ or $0.1 \times$ SSC ($0.5 \times$ SSC is sodium citrate, 7.5 mM; sodium chloride, 75 mM), hybridization was measured by exposing the membrane to photographic film (Hyperfilm-MP; Amersham).

Ability of strains to hydrolyse albumin. This was measured in two ways. Bacteria were grown on Columbia-blood agar, and the growth was harvested and suspended in 50 mM-Tris/HCl buffer (pH 7.5) to an OD₆₂₀ of $0.1 (1 \times 10^8 \text{ cells ml}^{-1})$. The bacterial suspension (50 µl) was mixed with 50 µl fluorescein-isothiocyanate-labelled bovine serum albumin (Sigma) and incubated for 24 h at 37 °C. After incubation the mixture was examined for an increase in fluorescence under UV light.

A second method was developed which allowed the hydrolysis of human serum albumin to be examined. Strains were grown in BHI and resuspended in PBS to an OD₆₆₀ of 1.0. The method then followed that for measuring binding to albumin (see above). After incubation of strains with ^{125}I -labelled albumin, the unbound albumin was removed by centrifugation and washing in PBS and the cells then boiled in 0.5% SDS to remove the bound albumin. The SDS-soluble material was then subjected to SDS-PAGE on 8–25% gradient gels, Western-blotted onto nitrocellulose and a film (Hyperfilm-MP; Amersham) was exposed to the blot. Controls of albumin alone were also run. The number of radiolabelled band(s) demonstrated the integrity or otherwise of the albumin.

Results

Biochemical characterization of strains

The species to which the various SMG strains belonged are listed in Table 1. Representatives of all three species within the SMG were identified and conformed to the published reactions (Beighton & Whiley, 1990; Whiley *et al.*, 1990). Three strains reacted with Lancefield group C antisera, two with Lancefield group G antisera, two were Lancefield group F positive and one was ungroupable.

Binding of albumin to strains

Table 1 demonstrates that Lancefield group C strains bound considerably more ($> 16 \times$) rat albumin than the other strains. The amount bound was not related to species or site of isolation. The ability to bind albumin was found to be a stable characteristic as repeated subculture ($30 \times$) of strain C5 did not reduce the amount of albumin bound.

S. intermedius strain C5 was selected for further study into the types of albumins bound by the group C strains of SMG. As can be seen in Fig. 1, C5 bound considerably more human, monkey, baboon, dog, cat, mouse, hamster and guinea-pig albumin than horse, rabbit, pig, sheep, cow, goat or chicken albumin, with rat and donkey albumin giving intermediate values. To determine whether this specificity was possibly a general feature of group C SMG, the binding of rat, human, rabbit and cow albumins was measured; all the group C strains gave similar results (Table 2). Table 2 also demonstrates that *S. anginosus* F4 (group F) and G14 (group G) did not

bind large amounts of any of the albumins tested, whereas the large-colony group C and G streptococci all bound albumins in the order human $>$ rat $>$ cow $>$ rabbit, and, in general, strain G148 bound more albumin than the others.

Localization of albumin receptor

The albumin-gold conjugate was located very near the cell surface for all three Lancefield group C SMG (Fig. 2). The control, albumin-gold interaction with *S.*

Fig. 1. Binding of albumin to *S. intermedius* C5 Lancefield group C. The bars represent standard deviation about the mean.

Table 2. Ability of streptococcal strains to bind to different albumin types

Strain*	Albumin bound (molecules per cell) $\pm$ SD†			
	Rat	Human	Rabbit	Cow
POW1 (C)	2768 $\pm$ 176	8232 $\pm$ 70	37 $\pm$ 7	244 $\pm$ 38
C117221-1 (C)	2553 $\pm$ 172	7092 $\pm$ 34	14 $\pm$ 4	131 $\pm$ 44
C5 (C)	2828 $\pm$ 137	7488 $\pm$ 116	75 $\pm$ 5	180 $\pm$ 31
F4 (F)	155 $\pm$ 17	28 $\pm$ 4	11 $\pm$ 3	13 $\pm$ 4
G14 (G)	101 $\pm$ 23	8 $\pm$ 3	–1.7 $\pm$ 7	4 $\pm$ 3
G23 (G)	818 $\pm$ 141	204 $\pm$ 59	13 $\pm$ 4	22 $\pm$ 8
G148 (G)‡	1919 $\pm$ 70	8173 $\pm$ 307	83 $\pm$ 25	112 $\pm$ 88
G43 (G)‡	1764 $\pm$ 138	4392 $\pm$ 448	14 $\pm$ 6	116 $\pm$ 15
C49 (C)‡	1295 $\pm$ 150	5532 $\pm$ 559	62 $\pm$ 6	117 $\pm$ 63

* Serological group in parentheses.

† 1×10^{-11} mol ^{125}I -labelled albumin added to 5×10^8 cells.

‡ Large-colony streptococci, not SMG.

Fig. 2. Electron micrographs of albumin-colloidal-gold-labelled Lancefield group C *S. milleri* strains. (a) Strain C5; (b) strain C117221-1; (c) strain POW1. The gold particles are close to the surface of the cells. Bars, 50 nm.

Table 3. Effect of various treatments on the ability of strain C5 to bind albumin

The values are the means of six replicates. Standard deviations were always less than 20% of the mean values.

Treatment	Rat albumin bound (mols per cell)
None	2828
Proteinase K	368*
Lipase	2687
Periodate	2319
IgG (non-immune)	2545
IgG to P1	3422
IgG to LTA	3394
IgG to group C polysaccharide	2970
Human albumin	961*
Bovine albumin	1607*
Ovalbumin	2513

* Significant ($P < 0.01$) effect of the treatment on the ability of cells to bind albumin.

anginosus F4, did not show any gold particles on grids (data not shown). The photomicrograph of POW1 (Fig. 2c) shows a protuberance or tuft on the cell surface. It can be seen that only one albumin-gold conjugate

adhered to this part of the cell, whereas far more albumin-gold adhered to the rest of the cell surface.

Factors affecting albumin binding

The effects of various procedures on the cell surface of strain C5 were examined with a view to determining the molecular identity of the albumin receptor (Table 3). Proteinase K reduced the binding to rat albumin, whereas lipase and periodate treatment of cells did not reduce binding, indicating that binding does not involve lipid or carbohydrate. IgG to group C polysaccharide, *S. mutans* protein P1, LTA, or non-immune IgG similarly had no effect. Human and, to a lesser extent, cow albumins were able to compete with the labelled rat albumin for the receptor, whereas ovalbumin was ineffective.

Extraction of receptor

Of the various methods that were tested for their ability to extract a functional receptor, only incubation for 18 h with lysozyme was effective. After SDS-PAGE, Western blotting and autoradiography, the M_r of the receptor was estimated to be 24000. Only one band was seen after

Fig. 3. SDS-PAGE of lysozyme-extracted proteins from strain C5 after affinity chromatography on human albumin-Sepharose. Lanes: 1, fraction eluted with 0.2 M-glycine/HCl, pH 2.6; 2, fraction eluted with 1 M-NaCl; 3, void volume; 4, lysozyme-treated C5 prior to affinity chromatography. The positions of molecular mass markers are indicated on the left. The arrow indicates the position of the receptor for ^{125}I -labelled albumin, approximate M_r 24000.

autoradiography, indicating that the strains possessed only one receptor type for albumin.

Purification of receptor

Fig. 3 shows the SDS-PAGE profiles of the peaks obtained after affinity chromatography and the position of the band visible on autoradiographs. A number of proteins eluted in the void volume; 1 M-NaCl eluted a major component of M_r 18000; elution with 1 M- or 4 M-urea did not elute any component. Glycine/HCl buffer (pH 2.6) eluted several components with two major bands of M_r 24000 and 14000. The autoradiograph demonstrated that the albumin receptor was eluted with glycine/HCl and was localized to the 24000 protein band.

Characterization of purified receptor

A comparison of radioactivity associated with the cells with and without added receptor showed that a 75% reduction in albumin binding had occurred in the presence of the receptor. Incubation of the receptor with insoluble trypsin at 37 °C destroyed its ability to inhibit albumin binding, which is consistent with it being a protein. The purified receptor failed to bind ^{125}I -IgG as shown by the lack of reactive bands after autoradiography.

Test for similarity to protein G

The possible relation between the albumin receptors of SMG and protein G of large-colony group G *Streptococcus* was investigated. An oligonucleotide probe was

synthesized and used to probe chromosomal DNA of SMG. None of the SMG strains showed reactivity with the probe in conditions of high or low stringency, which may indicate that they do not possess the identical gene for the protein G albumin-binding region (data not presented). The only strain that did hybridize with the probe was large-colony group G strain G148.

Hydrolysis of albumin

Two methods were developed to determine whether SMG strains were able to hydrolyse albumin, as enzymes may function as receptors. In neither case could any evidence of hydrolysis be detected. No SMG strain showed an increase in fluorescence after incubation of cells for 18 h at 37 °C with fluorescein-labelled albumin. Similarly, when the Lancefield group C SMG strains were incubated with ^{125}I -human albumin for 30 min and the albumin removed from the cell surface by boiling in detergent, no evidence of albumin degradation could be shown after SDS-PAGE and autoradiography of the extract.

Discussion

This study has demonstrated that SMG strains that react with Lancefield group C antisera are able to bind large amounts of certain types of albumin to their cell surface. No other SMG strain was able to do this to any great extent and the reaction was independent of the species within the SMG. It has been reported that Lancefield group C strains account for approximately 9% of all SMG isolates (Gossling, 1988; Whiley *et al.*, 1990). This may be an underestimate as a common practice in routine bacteriology laboratories is to differentiate β -haemolytic streptococci solely on their possession of Lancefield group antigens and therefore group C SMG would be reported simply as group C streptococci. Indeed, 56–75% of all group C streptococci isolated from infections are SMG (Lawrence *et al.*, 1985; Lebrun *et al.*, 1986; Ruoff *et al.*, 1985). While there has been only one report on the binding of albumin (cow) by SMG (Willcox *et al.*, 1992), the ability of other streptococci carrying Lancefield group C and G antigens, namely *S. dysgalactiae*, *S. canis*, *S. zooepidemicus*, *S. equi* and *S. equisimilis*, to bind albumin has been studied in some detail (Sjöbring *et al.*, 1991; Wideback & Kronvall, 1982; Raeder *et al.*, 1991; Lämmeler *et al.*, 1988; Otten & Boyle, 1991); five or more different types of albumin receptor have been described which are differentiated by their ability to bind specific albumin types. The albumin receptor on SMG strains may be related to 'type a' receptor found on *S. equisimilis* and human group G

streptococci as both types bind human, mouse and dog albumin but not rabbit or cow albumin.

The ability of SMG strains to bind different albumins is probably the property of a single cell-surface component, as human albumin and, to a lesser extent, cow albumin could inhibit the binding of rat albumin to the bacteria. The inhibition seen with cow albumin indicates that the low level of binding was of a specific nature. The receptor for albumin was shown to be a protein. It could be removed from the cell surface by proteinase digestion of whole cells, stained with Coomassie blue after purification on albumin-Sepharose, and the purified receptor lost its ability to inhibit the binding of labelled albumin to the surface of the SMG after digestion with proteinase K. Albumin-gold labelling of the surface of the streptococci revealed that the albumin receptor was situated very close to the cell wall. Tufts and fimbriae have been demonstrated on the surface of SMG (Willcox & Drucker, 1989; unpublished data), but it appears unlikely that the receptor is associated with these structures.

SDS-PAGE of the purified receptor and of lysozyme-extracted receptor revealed that its M_r was 24000. This value was less than the M_r values of albumin receptors extracted from large-colony group C or G streptococci, which range from 48000–51000 (Sjöbring, 1992; Lämmler *et al.*, 1988). The oligonucleotide probe synthesized to match a conserved region of the albumin-binding domain to protein G, an albumin 'type a' and IgG binding molecule of large-colony group C and G streptococci, showed no homology to total DNA of SMG; this is consistent with previous reports that SMG cannot bind to IgG (Lebrun *et al.*, 1986) and may indicate that the SMG receptor is not protein G but a unique albumin-binding molecule. The G+C ratio of SMG is 37–42 mol % (Drucker & Lee, 1983; Ezaki *et al.*, 1986) and of large-colony group C (*S. equi*, *S. zooepidemicus*, *S. dysgalactiae* and *S. equisimilis*) and large-colony group G streptococci is 37–40 mol % (Ezaki *et al.*, 1986). These similar values may indicate that the non-homology with the oligonucleotide is not the result of different codon usage between the groups. Albumin receptors, and many IgG receptors, can be removed from streptococci by boiling at acidic pH (Raeder *et al.*, 1991; Lämmler *et al.*, 1988). In contrast, the albumin receptor of strain C5 was either not released by boiling at acidic pH or was destroyed as no reactive bands could be seen after SDS-PAGE and autoradiography. This finding also points to differences between the receptor on the SMG and other streptococcal albumin receptors. Glycine/HCl buffer eluted more than one band from the affinity column, which may be the result of degradation of the receptor by the low pH of the buffer, and this may parallel the inability of acid to liberate a functional

receptor from the surface of cells. Indeed, an overloaded SDS-PAGE gel of the purified receptor showed considerable 'laddering', with bands being detected between M_r 24000 and 14000 (data not shown).

It has been previously demonstrated that lipoteichoic acid (LTA) can bind to albumin via its lipid component (Simpson *et al.*, 1980). The demonstration that lipase digestion of the cell surface did not reduce albumin binding indicates that lipids were not involved in the binding. The addition of Tween 20 to the buffer system which can elute LTA from albumin (Simpson *et al.*, 1980), and the fact that group F SMG possess LTA-like material (Whitworth *et al.*, 1992) but did not bind large amounts of albumin, makes it unlikely that LTA contributes to albumin binding. Enzymes can act as receptors and a report has demonstrated that SMG strains can degrade bovine albumin (Homer *et al.*, 1990). Under the growth conditions employed in this study no SMG strain was able to degrade bovine or human albumin, and it seems unlikely therefore that the protein responsible for albumin binding also has enzymic ability.

In conclusion, the data presented in this study indicate that certain SMG strains are able to bind to albumin and that the cell-surface receptor for albumin of a strain of *S. intermedius* has an M_r of 24000. Further work is currently in progress to isolate the gene encoding the receptor for albumin and to make mutants of SMG strains that lack albumin-binding ability, to assess the possible role of binding as a pathogenic determinant.

This research was supported by grants from the NHMRC, the Dental Board of New South Wales and the Australian Dental Research Fund Inc.

References

- BEIGHTON, D. & WHILEY, R. A. (1990). Sialidase activity of the 'Streptococcus milleri group' and other viridans streptococci. *Journal of Clinical Microbiology* **28**, 1431–1433.
- BESLEY, J. E. (1989). *Colloidal Gold: a New Perspective for Cytochemical Marking*, p. 10. Oxford: Oxford University Press.
- CIMOLAI, N., ELDFORD, R. W., BRYAN, L., ANAND, C. & BERGER, P. (1988). Do the β -hemolytic non-group A streptococci cause pharyngitis? *Reviews of Infectious Diseases* **10**, 587–601.
- DRUCKER, D. B. & GREEN, R. M. (1978). The relative cariogenicities of *Streptococcus milleri* and other viridans group streptococci in gnotobiotic hooded rats. *Archives of Oral Biology* **23**, 183–187.
- DRUCKER, D. B. & LEE, S. M. (1983). Possible heterogeneity of *Streptococcus milleri* determined by DNA mol % (guanine plus cytosine) measurement and physiological characterization. *Microbios* **38**, 151–157.
- ERICSON, D., BJÖRCK, L. & KRONVALL, G. (1980). Further characteristics of β_2 -microglobulin binding to oral streptococci. *Infection and Immunity* **30**, 117–124.
- EZAKI, T., FACKLAM, R., TAKEUCHI, N. & YABUCHI, E. (1986). Genetic relatedness between the type strain of *Streptococcus anginosus* and minute-colony-forming beta-hemolytic streptococci carrying different Lancefield grouping antigens. *International Journal of Systematic Bacteriology* **36**, 345–347.
- FINLAY-JONES, J. J., HART, P. H., SPENCER, L. K., NULSEN, M. F., KENNY, P. A. & McDONALD, P. J. (1991). Bacterial killing *in vitro* by

- abscess-derived neutrophils. *Journal of Medical Microbiology* **34**, 73–81.
- FORESTER, H., HUNTER, N. & KNOX, K. W. (1983). Characteristics of a high molecular mass extracellular protein of *Streptococcus mutans*. *Journal of General Microbiology* **129**, 2779–2788.
- GOSSLING, J. (1988). Occurrence and pathogenicity of the *Streptococcus milleri* group. *Reviews of Infectious Diseases* **10**, 257–285.
- HOMER, K. A., WHILEY, R. A. & BEIGHTON, D. (1990). Proteolytic activity of oral streptococci. *FEMS Microbiology Letters* **67**, 257–260.
- JENKINSON, H. F. (1986). Cell-surface proteins of *Streptococcus sanguis* associated with cell hydrophobicity and coaggregation properties. *Journal of General Microbiology* **132**, 1575–1589.
- LÄMMLER, C. & STING, R. (1989). Interaction of type II IgG Fc-receptors from streptococci of serological group A with murine IgG. *Zentralblatt für Bakteriologie* **271**, 460–465.
- LÄMMLER, C., SCHAUFUSS, P., FREDE, C. & BLOBEL, H. (1987). Binding of plasma proteins to streptococci of serological group L with special reference to their immunoglobulin G Fc-receptor activity. *Canadian Journal of Microbiology* **34**, 1–5.
- LÄMMLER, C., FREDE, C., GURTURK, K., HILDEBRAND, A. & BLOBEL, H. (1988). Binding activity of *Streptococcus canis* for albumin and other plasma proteins. *Journal of General Microbiology* **134**, 2317–2323.
- LAWRENCE, J., YAJKO, D. M. & HADLEY, W. K. (1985). Incidence and characterisation of beta-hemolytic *Streptococcus milleri* and differentiation from *S. pyogenes* (group A), *S. equisimilis* (group C), and large-colony group G streptococci. *Journal of Clinical Microbiology* **22**, 772–777.
- LEBRUN, L., GUIBERT, M., WALLET, P., DE MANEVILLE, M.-M. & PILLOT, J. (1986). Human Fc(γ) receptors for differentiation in throat cultures of group C '*Streptococcus equisimilis*' and group C '*Streptococcus milleri*'. *Journal of Clinical Microbiology* **24**, 705–707.
- MARKWELL, M. A. K. (1982). A new solid-state reagent to iodinate proteins. *Analytical Biochemistry* **125**, 427–432.
- MEJÄRE, B. & EDWARDSSON, S. (1975). *Streptococcus milleri* (Guthof); an indigenous organism of the human oral cavity. *Archives of Oral Biology* **20**, 757–762.
- MJÖRNER, H., MYHRE, E., BJÖRCK, L. & KRONVALL, G. (1980). Effect of specific binding of human albumin, fibrinogen, and immunoglobulin G on surface characteristics of bacterial strains as revealed by partition experiments in polymer phase systems. *Infection and Immunity* **29**, 879–885.
- MOORE-GILLON, J. C., EYKYN, S. J. & PHILLIPS, I. (1981). Microbiology of pyogenic liver abscess. *British Medical Journal* **283**, 819–821.
- OTTEN, R. A. & BOYLE, M. D. P. (1991). Characterisation of protein G expressed by human group C and G streptococci. *Journal of Microbiological Methods* **13**, 185–200.
- RAEDER, R., OTTEN, R. A. & BOYLE, M. D. P. (1991). Comparison of albumin receptors expressed on bovine and human group G streptococci. *Infection and Immunity* **59**, 609–616.
- RUOFF, K. L. (1988). *Streptococcus anginosus* ('*Streptococcus milleri*'): the unrecognised pathogen. *Clinical Microbiology Reviews* **1**, 102–108.
- RUOFF, K. L., KUNZ, L. J. & FERRARO, M. J. (1985). Occurrence of *Streptococcus milleri* among beta-hemolytic streptococci isolated from clinical specimens. *Journal of Clinical Microbiology* **22**, 149–151.
- SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989). *Molecular Cloning, A Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- SANDGREN, C. H., MAMO, W., LARSSON, I., LINDAHL, M. & BJÖRK, I. (1991). A periodate-sensitive anti-phagocytic surface structure, induced by growth in milk whey, on *Staphylococcus aureus* isolated from bovine mastitis. *Microbial Pathogenesis* **11**, 211–220.
- SIMPSON, W. A., OFEK, I. & BEACHEY, E. H. (1980). Binding of streptococcal lipoteichoic acid to the fatty acid binding sites on serum albumin. *Journal of Biological Chemistry* **255**, 6092–6097.
- SJÖBRING, U. (1992). Isolation and molecular characterisation of a novel albumin-binding protein from group G streptococci. *Infection and Immunity* **60**, 3601–3608.
- SJÖBRING, U., BJÖRCK, L. & KASTERN, W. (1991). Streptococcal protein G: gene structure and protein binding properties. *Journal of Biological Chemistry* **266**, 399–405.
- SMITH, R. F. & WILLET, N. P. (1968). Rapid plate method for screening hyaluronidase and chondroitin sulfatase-producing microorganisms. *Applied Microbiology* **16**, 1434–1436.
- SWITALSKI, L. M., MURCHISON, H., TIMPL, R., CURTISS III, R. & HOOK, M. (1987). Binding of laminin to oral and endocarditis strains of viridans streptococci. *Journal of Bacteriology* **169**, 1095–1101.
- ULLBERG, M., KRONVALL, G. & WIMAN, B. (1989). New receptor for human plasminogen on gram positive cocci. *APMIS* **97**, 996–1002.
- WAGNER, B., SCHMIDT, K.-H., WAGNER, M. & KÖHLER, W. (1986). Albumin bound to the surface of M-protein positive streptococci increased their phagocytosis by polymorphonuclear leukocytes in the absence of complement and bactericidal antibodies. *Zentralblatt für Bakteriologie, Mikrobiologie und Hygiene, Series A* **261**, 432–446.
- WHILEY, R. A. & HARDIE, J. M. (1989). DNA-DNA hybridization studies and phenotypic characteristics of strains within the '*Streptococcus milleri*' group. *Journal of General Microbiology* **135**, 2623–2633.
- WHILEY, R. A., FRASER, H., HARDIE, J. M. & BEIGHTON, D. (1990). Phenotypic differentiation of *Streptococcus intermedius*, *Streptococcus constellatus*, and *Streptococcus anginosus* strains within the '*Streptococcus milleri* group'. *Journal of Clinical Microbiology* **28**, 1497–1501.
- WHITWORTH, J. M., POXTON, I. R. & ROSS, P. W. (1992). Immunochemistry of the membrane associated carbohydrate antigens of group F and related streptococci (*S. milleri*). In *New Perspectives on Streptococci and Streptococcal Infections* (Zentralblatt für Bakteriologie suppl. 22), pp. 219–220. Edited by G. Orefici. Stuttgart: Gustav Fischer Verlag.
- WICKEN, A. J. & KNOX, K. W. (1971). A serological comparison of the membrane teichoic acids from lactobacilli of different serological groups. *Journal of General Microbiology* **67**, 251–254.
- WIDEBACK, K. & KRONVALL, G. (1982). Surface receptors for serum albumin in group C and G streptococci show three different types of albumin specificity. *Infection and Immunity* **38**, 1154–1163.
- WIDEBACK, K., SEAL, U. S. & KRONVALL, G. (1982). Receptor in group C and G streptococci detects albumin structures present in mammalian species. *Infection and Immunity* **36**, 469–475.
- WILLCOX, M. D. P. & DRUCKER, D. B. (1989). Surface structures, coaggregation and adherence phenomena of *Streptococcus oralis* and related species. *Microbios* **59**, 19–29.
- WILLCOX, M. D. P. & KNOX, K. W. (1990). Surface-associated properties of *Streptococcus milleri* group strains and their potential relation to pathogenesis. *Journal of Medical Microbiology* **31**, 259–270.
- WILLCOX, M. D. P., GEYELIN, A. J. & KNOX, K. W. (1992). The binding of fibronectin and albumin to strains within the *Streptococcus milleri* group. In *New Perspectives on Streptococci and Streptococcal Infections* (Zentralblatt für Bakteriologie suppl. 22), pp. 154–155. Edited by G. Orefici. Stuttgart: Gustav Fischer Verlag.
- WILLIAMS, B. L., MCCANN, G. F. & SCHOENKNECHT, F. D. (1983). Bacteriology of dental abscesses of endodontic origin. *Journal of Clinical Microbiology* **18**, 770–774.
- WYATT, J. E., WILLCOX, M. D. P., RUSSELL, R. R. B. & HANDLEY, P. S. (1988). Fibrillar strains of *Streptococcus sanguis* biotype I carry a surface protein which cross-reacts with antigen B from *Streptococcus mutans* Ingbritt. *Oral Microbiology and Immunology* **3**, 162–168.