Adherence of glucan-positive and glucan-negative strains of *Streptococcus bovis* to human epithelial cells

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Summary. Adherence to buccal epithelial cells (BEC) and the role played in the binding by lipoteichoic acid (LTA) and other superficial components have been studied in reference and clinical strains of *Streptococcus bovis* either glucan-positive biotype I or glucan-negative biotype II. To avoid the synthesis of glucan by biotype I strains, adherence was studied in bacteria grown in Todd-Hewitt broth, a sucrose deficient medium. Both biotypes were shown to bind to BEC and clinical isolates, irrespective of biotype attached to the same degree but in greater numbers than reference strains. Inhibition studies suggest that at least two mechanisms, —LTA and protein-mediated—are responsible for the adherence of both glucan-positive and negative strains of *S. bovis*. Moreover, in glucan-positive strains capsular polysaccharides may be also involved.

Introduction

*Streptococcus bovis* is a well-known cause of endocarditis in elderly people. Bacteraemia due to *S. bovis* has been associated mostly with underlying colonic neoplasms and to a lesser extent with gastrointestinal tract and oropharyngeal carcinoma. Although *S. bovis* can be isolated from the faeces of normal subjects, Klein et al. found that in patients with carcinoma of the colon the isolation rate of *S. bovis* from faecal cultures could be as high as 56%. Two biotypes of *S. bovis* have been recognised on the basis of physiological and biochemical characteristics—biotype I, which ferments mannitol, hydrolyses starch and produces glucan from sucrose, and biotype II (or *S. bovis* variant) which gives negative results in all these tests.

The capacity to produce soluble glucan is an important factor in mediating the adherence of *S. bovis* to damaged valve endothelium. Glucan-positive strains are isolated more frequently than glucan-negative strains from patients with endocarditis or with malignant colonic lesions, although the role played by capsular components such as lipoteichoic acid (LTA) or extracellular products such as glucan has not been elucidated. LTA is known to be involved in the attachment of staphylococci to host cell tissue and of group A streptococci and group B streptococci. By immuno-electronmicroscopy, LTA has been located in the capsule of *S. bovis* with an arrangement similar to that seen in other streptococci. In a previous report we showed that both glucan-positive and glucan-negative reference strains of *S. bovis* were able to adhere to the oral mucosa, a finding that prompted us to evaluate the adherence of clinical and reference strains of *S. bovis* to human buccal epithelial cells (BEC) and to study the possible role of capsular components such as LTA in the binding of both biotypes to mucosal surfaces.

Materials and methods

Bacteria

*Streptococcus bovis* NCTC 8177 biotype I, glucan-positive, and *S. bovis* NCTC 11436 biotype II, glucan-negative, were kindly supplied by Dr G. Colman (Central Public Health Laboratory, Colindale, London). Clinical strains of both biotypes isolated from the blood of patients with endocarditis were identified by API20-STREP (bioMerieux, France) and checked for glucan production by the ethanol precipitation method of Hehre and Neill. Briefly, strains were grown for 48 h in Brain Heart Infusion Broth (Unipath) supplemented with glucose 8.5% w/v for the control or saccharose for glucan production; cultures were centrifuged and 0.25 ml of the supernates were added to 2.25 ml of sodium acetate 10%. After addition of 6 ml of ethanol, glucan-positive cultures gave a heavy precipitate in the saccharose but not in the glucose medium.

Organisms were lyophilised and stored. For adherence tests, strains, freshly passaged on to sheep blood 5% v/v Columbia Agar (Unipath), were grown overnight in Todd-Hewitt Broth (Unipath) at 37°C.
Adherence assay

BEC were obtained from healthy non-smoking adults by gently scraping the oral mucosa with a sterile cotton swab. Cells were washed with cold sterile phosphate buffered saline (PBS; 0.02 M phosphate, 0.15 M NaCl, pH 7.4) with 12-μm membrane filters (Sartorius, Italy). One ml (10^6 cells) of BEC suspended in PBS was mixed with 1 ml of S. bovis suspension (10^8 cfu/ml) and incubated with agitation at 37°C for 1 h. The cells were then washed by membrane filtration with PBS to eliminate unattached bacteria. Direct smears were prepared from each BEC suspension and stained with crystal violet for 15 s. Numbers of chains attached to at least 50 BEC were enumerated by light microscopy, each chain being counted as a single cfu. Epithelial cells with > 50 adherent chains were counted as having 50.

Bacterial and BEC treatments

Two mls of the standard bacterial suspension were added to one of the following. (1) Antisera raised against S. bovis biotype I, biotype II, anti-LTA or anti-glucan sera: bacterial pellets collected by centrifugation were directly suspended in a 1 in 50 dilution of the serum and incubated for 1 h at 37°C. (2) Sodium metaperiodate: pellets were suspended in PBS containing sodium metaperiodate 5% w/v and incubated for 10 min under nitrogen at room temperature. (3) Pepsin: bacteria were incubated for 30 min at 37°C in a 50 mM citrate-10 mM phosphate buffer, pH 3, containing pepsin (Merck, Darmstadt, Germany; activity, 2500 FIP-U/g) 1.5 mg/ml; the control was incubated in the same buffer without pepsin. (4) Trypsin: bacteria were digested with trypsin (Type XIII, Sigma) at a final concentration of 80 U/ml at 37°C for 1 h; the activity of the trypsin was then stopped by adding soybean trypsin inhibitor (200 U/ml; Sigma). (5) Bovine serum albumin (BSA; Sigma): bacteria were incubated for 1 h at 37°C in PBS containing BSA 1.5 mg/ml. After these additions bacterial suspensions were centrifuged, washed twice in PBS, passed through a tuberculosis needle to reduce clumping, and assayed for adherence to BEC.

Pre-treatments of BEC were performed by incubating the cells for 1 h at 37°C with various concentrations of LTA or glucan. LTA was prepared by phenol extraction from the supernate of S. bovis cultures supplemented with penicillin and purified by Sepharose CL-6B chromatography as previously reported. Glucan was extracted from S. bovis NCTC 8177 (biotype I) culture supernates according to the method of Ramirez-Rondà and purified by Sepharose 4B gel filtration. After purification, the glucan preparation comprised 90% glucose as determined by the phenol-sulphuric acid method.

Immune sera

Serum against purified LTA was raised in rabbits according to the method of Fiedel and Jackson and its titre, determined by passive haemagglutination assay, was 256. Briefly, 400 μl of a suspension of sheep erythrocytes 5% v/v were passively sensitised for 1 h at 37°C with 400 μl of solution of LTA 1 mg/ml. For the assay, 50 μl of sensitised erythrocytes suspended at 2% v/v in PBS were added to 100 μl of anti-LTA serum that was diluted two-fold. Agglutination was read after incubation for 2 h at 37°C.

Anti-glucan serum was prepared in rabbits immunised intravenously weekly for 5 weeks with 4 ml of an emulsion of glucan 3.5 mg in PBS 1 ml, 1 ml of incomplete Freund's adjuvant and 2 ml of saline containing Tween 80 2% v/v. When tested by ELISA with glucan 1 μg/well used for the coating, the titre achieved was 4000.

Anti-biotype sera, raised in rabbits against S. bovis NCTC 8177 for biotype I and S. bovis NCTC 11436 for biotype II, were prepared by intravenous injection three times weekly for 6 weeks of 1 ml of a formalinised bacterial suspension derived from 500 ml of a culture suspended in 20 ml of saline. Titres of anti-S. bovis sera were determined by ELISA, coating the plate wells overnight with 100 μl of bacterial suspension (OD530nm 0.33) in carbonate buffer. After blocking with 100 μl of non-fat dry milk 5% w/v solution, the test was performed by ELISA. Titres of anti-S. bovis biotype I and biotype II were 64000 and 32000, respectively.

Statistical analysis

Differences in adherence between control and treated conditions were analysed for significance by Student's t test.

Results

Adherence of glucan-positive and glucan-negative strains of S. bovis

The adherence of fresh clinical isolates of S. bovis in comparison with reference strains from stock cultures was one of our initial investigations. Both glucan-positive biotype I and glucan-negative biotype II strains were studied. The results of these experiments are shown in table I. Although all strains showed marked adherence to BEC in the standard assay, a marked difference in adherence between the clinical isolates and the reference strains was observed. In four experiments, the mean number of adherent bacteria for strain NCTC 8177 biotype I, the reference strain, was 15 (SEM 1.08), whereas a clinical isolate, strain 595 of the same biotype gave a count of 36 (SEM 2.2). Similarly the clinical isolate belonging to biotype II, strain 642, produced higher counts than the reference strain NCTC 11436—34 (SEM 2.41) and 6 (SEM 0.86), respectively (table I).
Table I. Adherence to human BEC of glucan-positive (biotype I) and glucan-negative (biotype II) strains of *S. bovis*

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Biotype</th>
<th>Source</th>
<th>Adherence*</th>
<th>Expt 1</th>
<th>Expt 2</th>
<th>Expt 3</th>
<th>Expt 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCTC 8177</td>
<td>I</td>
<td>Reference strain</td>
<td>15 (1.42)</td>
<td>13 (1.14)</td>
<td>18 (1.43)</td>
<td>14 (1.57)</td>
<td></td>
</tr>
<tr>
<td>NCTC 11436</td>
<td>II</td>
<td>Reference Strain</td>
<td>8 (1.00)</td>
<td>5 (0.43)</td>
<td>6 (0.86)</td>
<td>4 (0.57)</td>
<td></td>
</tr>
<tr>
<td>595</td>
<td>I</td>
<td>Clinical isolate</td>
<td>39 (3.14)</td>
<td>40 (2.14)</td>
<td>37 (3.42)</td>
<td>30 (3.71)</td>
<td></td>
</tr>
<tr>
<td>1769</td>
<td>I</td>
<td>Clinical isolate</td>
<td>34 (3.42)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>1739</td>
<td>II</td>
<td>Clinical isolate</td>
<td>30 (3.14)</td>
<td>32 (2.71)</td>
<td>33 (3.42)</td>
<td>41 (4.00)</td>
<td></td>
</tr>
<tr>
<td>642</td>
<td>I</td>
<td>Clinical isolate</td>
<td>45 (3.42)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

* Adherence is expressed as the mean (SEM) number of streptococcal chains adhering/cell. The mean was the average number of bacteria adhering to 50 cells.

Table II. Effect of pre-treatment of human BEC with LTA on the adherence of glucan-positive and glucan negative strains of *S. bovis*

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Biotype</th>
<th>Adherence* after treatment with LTA (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>595</td>
<td>I</td>
<td>40 (2.14) 38 (2.86) 41 (2.57) 32 (2.28)† 30 (2.14)† 34 (3.30)</td>
</tr>
<tr>
<td>642</td>
<td>II</td>
<td>32 (2.71) 25 (2.00) 24 (2.43) 23 (2.43)† 20 (2.00)† 34 (3.30)</td>
</tr>
</tbody>
</table>

* Mean (SEM) number of streptococcal chains/epithelial cell. Data are from three experiments.
† LTA was pre-incubated overnight at 4°C with anti-LTA serum diluted 1 in 50 and successively incubated with BEC.
‡ Value significantly lower than controls (p < 0.05). (p < 0.05) was observed (table II). The highest concentration of LTA tested was 500 µg/ml and at this level the degree of inhibition achieved was 25% for strain 595 and 37% for strain 642. This inhibition was abolished by pre-incubation of LTA with immune anti-LTA serum (table II). Similar inhibition values were obtained when BEC were pre-incubated with LTA extracted from group B streptococci. Conversely, pre-treatment of BEC with different amounts of purified glucan did not inhibit significantly the adherence either of the glucan-positive or glucan-negative strain.

Effect of pre-treatment of BEC with LTA or glucan on adherence

When BEC were pre-treated with purified *S. bovis* LTA a low but significant dose-dependent inhibition was observed (table II). The highest concentration of LTA tested was 500 µg/ml and at this level the degree of inhibition achieved was 25% for strain 595 and 37% for strain 642. This inhibition was abolished by pre-incubation of LTA with immune anti-LTA serum (table II). Similar inhibition values were obtained when BEC were pre-incubated with LTA extracted from group B streptococci. Conversely, pre-treatment of BEC with different amounts of purified glucan did not inhibit significantly the adherence either of the glucan-positive or glucan-negative strain.

Effect of antibody treatment on adherence

The pre-treatment of reference strains or clinical isolates of *S. bovis* with anti-LTA serum showed a significantly reduced degree of adherence of both biotypes, reaching a maximum of 50% (figure). Anti-glucan serum inhibited the adherence of reference strains more than that of clinical isolates. Inhibition of strain NCTC 8177 biotype I was particularly high (69%), whereas the adherence of the clinical isolate of strain 642 biotype II was not affected (figure).

Pre-incubation of *S. bovis* strains with antisera raised against biotype I and biotype II reference strains showed that adherence of both biotypes could be partially inhibited by the corresponding anti-biotype serum. Anti-biotype II serum cross-reacted with the two biotype I strains, reducing their adherence to
BEC, albeit to a lower extent than with the homologous strains. In contrast, serum raised against biotype I did not reduce the adherence of the heterologous biotype (figure). Treatment of bacterial cells with a non-immune rabbit serum at a dilution of 1 in 50 did not affect the adherence to BEC of any strain of \textit{S. bovis}.

Effects of pre-treatment of \textit{S. bovis} cells on adherence

In further experiments, the effect of BSA, a substance known to bind the lipid portion of LTA\textsuperscript{19} was investigated. Pre-treatment of bacteria with BSA caused a 50\% reduction in adherence for strain 595 and 62\% for strain 642 compared with the control PBS-treated bacteria (table III). Treatment of the cells with sodium metaperiodate, a strong sugar oxidant affecting vicinal glycol groups, strongly inhibited the attachment to BEC of the glucan producer strain 595 only (table III). On the other hand, treatment with pepsin brought about a decrease of 85–90\% in adherence of both biotypes of \textit{S. bovis} (table III). Trypsin digestion was less effective in reducing the binding of the bacteria to BEC, inhibiting the adherence of strain 595 by c. 35\% and strain 642 by c. 60\% and strain 642 by c. 35\% (table III).

Discussion

It has been demonstrated that the event that initiates the pathogenesis of bacterial diseases is often related to the ability of bacteria to adhere to the surface of host cells.\textsuperscript{19,28} In cases of endocarditis, \textit{S. bovis} biotype I was isolated more frequently than biotype II,\textsuperscript{4} and it has been inferred that the higher adherence and pathogenicity of biotype I strains correlate with the production of glucan.\textsuperscript{11,12} Nonetheless, glucan production \textit{in vitro} and its relationship with endocarditis remains unexplained. Indeed, if the production of glucan is related to the presence of saccharose,\textsuperscript{21} the synthesis of this polysaccharide is unlikely after the streptococcus has entered the bloodstream. In our evaluation of the possible role of non-glucan constituents in adhesion of both biotypes of \textit{S. bovis}, we avoided the use of conditions leading to glucan production. Moreover, in a follow-up to a previous report,\textsuperscript{29} we used \textit{S. bovis} clinical isolates that presumably had greater adhesive properties than the laboratory strains, thus demonstrating that strains of \textit{S. bovis} from patients with endocarditis are much more adherent to BEC than reference strains. Furthermore, the extensive attachment of these clinical strains was independent of their ability to produce glucan. Therefore, our observations indicate that, in virulent \textit{S. bovis} strains, other components may be involved in the binding to human cells and it has been reported that LTA is important in mediating the adherence of other glucan-positive streptococci, such as \textit{S. mutans} and \textit{S. sanguis}, isolated from endocarditis cases.\textsuperscript{35} The results obtained by treating human cells with LTA, or bacteria with anti-LTA serum and BSA, indicate that LTA plays a role in the adherence of both biotypes of \textit{S. bovis}. Although only a moderate degree of inhibition was obtained with purified LTA, this was seen in at least three experiments with each biotype and shown to be dose-dependent and saturable at a dose of LTA 500 \textmu g/ml.

Pre-treatment of \textit{S. bovis} cells with proteolytic enzymes reduced adherence; indeed pepsin almost entirely blocked binding of both biotypes to BEC. Streptococci suspended in citrate-phosphate buffer with or without pepsin were still viable after treatment for 1 h. However, the extract obtained from pepsin-treated cells, showed only a very low inhibitory activity, perhaps due to the fact that pepsin removed the protein(s) in an inactive form of peptides—this is suggested by the absence of any protein bands in electrophoresis. Speziale \textit{et al.}\textsuperscript{26} also showed that some proteolytic treatments hydrolysed the staphylococcal receptor for fibronectin. Thus, our findings suggest that proteinaceous adhesins may participate or co-operate with LTA for effective adherence of \textit{S. bovis} to BEC. This suggestion is supported by the demonstration of a greater degree of inhibition of adhesion by \textit{S. bovis} by treatment with pepsin than with anti-LTA serum. A similar mechanism of binding, LTA- and protein-mediated, has recently been demonstrated for \textit{S. pyogenes}\textsuperscript{37} and a multi-step model of adhesion has been proposed for streptococci.\textsuperscript{38} Finally, as suggested by Semjen and Galli,\textsuperscript{29} capsular polysaccharide constituents may also play a role in the adhesion of \textit{S. bovis}. We observed that the adherence of biotype I was partially reduced by anti-glucan.
serum and almost abolished by metaperiodate, findings not observed with the biotype II, a non-glucan-producer.

The adherence of clinical isolates to BEC was independent of biotype and ability to produce glucan, but also by the higher degree of inhibition of the reference strains which, in regard to adherence to BEC, differ markedly from clinical strains.

We thank Professor A. Cassone, (Istituto Superiore di Sanita, Rome, Italy) for help in reviewing the manuscript. This work was supported in part by the CNR Target Project Biotechnology and Bioinstrumentation, grant no. 89.00262.70.

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