BINDING OF TYPE-III GROUP-B STREPTOCOCCI TO BUCCAL EPITHELIAL CELLS

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SUMMARY. A binding assay was used to study the attachment of type-III group-B streptococci (GBS) to buccal epithelial cells. Results indicate that an adhesin, with the characteristics of a protein, is the molecule at the streptococcal cell surface responsible for attachment to the buccal cells. The bacterial adhesin probably recognises a sugar on the surface of the mucosal cell, because periodate oxidation of the buccal cells caused a significant reduction in subsequent adherence of GBS. A sonicate of type-III GBS blocked the binding of the organism to buccal cells. The effects of physical and chemical modifications of the sonicate on its ability to prevent bacterial attachment are described; these corroborate the evidence gained from heat and periodate treatments of the buccal cells and GBS. Results suggest a lectin type of attachment mechanism for type-III GBS which can be blocked by N-acetyl-D-glucosamine, rather than attachment by means of a lipoteichoic acid as described for group-A streptococci.

INTRODUCTION

It is widely accepted that adherence of bacteria to mucosal cells is important in the initiation of many types of microbial disease (Gibbons and van Houte, 1971). Group-B streptococci (GBS) are an important cause of serious neonatal illness (Anthony and Okada, 1977), which has been related to vaginal colonisation with these organisations (Ferrieri, Cleary and Seeds, 1977); several authors have reported the kinetics of the binding reaction between isolated vaginal epithelial cells and GBS (Mårdh and Weström, 1976; Zawaneh *et al.*, 1979). It has also been shown that the degree of attachment between bacteria and vaginal cells is related to the hormonal status of the vaginal-cell donor, reaching a maximum near the time of ovulation (Botta, 1979). At present, little is known of the particular bacterial cell-surface components concerned in the binding of GBS to epithelial cells. In group-A streptococci, membrane lipoteichoic acid (LTA) at the surface of the bacteria, is responsible, at least in part, for their adherence to buccal epithelial cells (BEC) (Beachey, 1975). This paper describes features of the binding of GBS to BEC and indicates the nature of the adhesin on the GBS cell surface that mediates attachment of the organism to BEC.

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MATERIALS AND METHODS

Bacteria. Type-III GBS (strain NCTC11080) and group-A streptococcus (strain NCTC10085) were from the National Collection of Type Cultures, Colindale Avenue, London NW9 5HT and were grown in static culture for 18 h at 37°C in Todd-Hewitt Broth (Oxoid CM189).

Buccal cells were gently scraped with a wooden spatula from the inside of the cheek of a healthy male donor and suspended in 10 ml of Dulbecco's Phosphate-buffered Saline (PBS; Oxoid BR14a). The suspension was placed in a thin-walled test tube and treated in a sonic water bath (Model 6441A, Dawe Inst. Ltd, Concord Road, Western Avenue, London W3 0SD) for 1½ min to aid the removal of background commensal organisms. The cells were then sedimented at 200 *g* for 10 min and washed twice more in PBS. The washed pellet was suspended in 2 ml of PBS and a cell count performed in a Neubauer counting chamber. The suspension was finally adjusted to a concentration of $2-6 \times 10^4$ cells/ml by dilution with PBS.

Adherence assay. The extinction at 650 nm (E_{650}) of the 18-h culture of GBS was adjusted to 0.43 which, from a previously determined calibration curve, yielded 10^8 cfu/ml. The volume was noted. The bacteria were sedimented at 900 *g* for 20 min, washed once in PBS, then resuspended to 10^8 cfu/ml in the previously noted volume of Dulbecco's PBS + Dulbecco "B" solution (Oxoid SR39) 0.5 ml/100 ml of PBS.

Samples of bacterial suspension (1 ml) were mixed with equal volumes of buccal-cell suspension in small, flat-bottomed sample tubes; the stoppered tubes were rotated at 30 rpm at 37°C for 45 min. After incubation, each mixture was filtered through a 10-µm filter (Gelman Sciences Inc., 600 S. Wagner Road, Ann Arbor, Michigan; polypropylene filter, 13-mm diameter) held in a Millipore filter holder, mounted on the end of a 10-ml syringe. Each filter was washed with 30 ml of PBS, carefully removed from the filter holder and inverted onto a drop of PBS on a clean microscope slide. The filters were lifted off the slide after approximately 2 min. The slides were air-dried, fixed in methanol for 5 min, and finally stained by Gram's method. The stained smears were mounted in DPX (dibutyl thallate xylol) under coverslips.

Each mixture of bacteria and buccal cells was prepared in duplicate and the results were averaged. Counting was performed at a magnification of 400 and for each experiment the bacteria adherent to each of 100 separate buccal cells was counted. Statistical significance was tested by Student's *t* test.

Preparation of sonicate from GBS. GBS were grown for 18 h in 3 L of Todd-Hewitt broth, at 37°C. Bacteria were harvested by centrifugation at 20 000 *g* for 10 min, washed once in PBS (pH 7.4, 0.01M) and suspended in 20 ml of PBS. The resulting suspension was treated in 5-ml portions in the sonic waterbath for 5 min and the cells were then sedimented at 20 000 *g* for 10 min. The supernate was collected and dialysed against 0.005M tris-HCl buffer (pH 7.4) for 3½ h at 4°C. The material was then lyophilised.

Heat-treated sonicate was prepared by autoclaving at 121°C for 15 min. For periodate oxidation of the sonicate, a 2-ml sample (protein 140 µg/ml in 0.01M PBS, pH 7.4) was made 0.1M in sodium metaperiodate and left overnight at room temperature, in the dark. Excess periodate was destroyed by treatment with ethylene glycol, followed by lyophilisation. The sample was then re-dissolved in 2 ml of distilled water. A control sample was treated in the same way except that periodate was not added.

Preparation of membrane lipoteichoic acid. LTA was extracted from the cytoplasmic membrane of type-III GBS with cold 80% aqueous phenol and purified on a column of Sepharose 6B by the method of Coley, Duckworth and Baddiley (1975).

Pretreatment of GBS. Bacterial suspensions were treated with periodate for 5 min at room temperature in 4 ml of acetate buffer, pH 4.6, containing sodium meta-periodate 10 mg/ml. Control organisms were suspended in the same buffer but which did not contain periodate. The bacteria were then washed once in PBS before being resuspended in Dulbecco's PBS + "B" to the required concentration. Bacteria were either heated in a 75°C water bath for 30 min or in an autoclave to 121°C for 15 min.

Bacteria were treated with trypsin (E.C. 3.4.21.4, Type III Sigma Chemical Co.) at a concentration of 10 000 N-benzoyl-L-arginine ethyl ester units/ml in 46 mM tris-HCl (pH 7.5) containing 11.5 mM CaCl₂ for 1 h at 37°C by the method of Saunders and Miller (1980).

Pretreatment of bacteria with various sugars was by incubation, at 37°C, of washed cells with

the relevant sugar, dissolved in Dulbecco's PBS + "B". Bacteria were washed once in Dulbecco's PBS + "B" before the adherence assay.

Pretreatment of buccal cells. Washed BEC were treated with streptococcal sonicate by mixing the cells with the sonicate on a tube rotator at 37°C for 1 h. The buccal cells were then sedimented by centrifugation at 200 *g* for 10 min.

Buccal cells were treated with LTA 0.7 mg/ml in the same manner as was the sonicate. Periodate treatment of the buccal cells was performed in the same way as for the GBS.

Analytical procedures. Protein concentration was estimated by the method of Lowry *et al.* (1951).

Polyacrylamide-gel electrophoresis (PAGE) was done in the presence of 1% sodium dodecyl sulphate (SDS) on 10% polyacrylamide slab gels with the discontinuous buffer system of Laemmli and Favre (1973). The gels were stained for protein with Coomassie brilliant blue R. A mixture of reference proteins (BDH, Poole, Dorset BH12 4NN) containing cytochrome C (mol. wt 12 300), myoglobin (17 200), chymotrypsinogen A (25 700), ovalbumin (45 000), albumin (66 200) and ovotransferrin (76–78 000) was used as an indicator of molecular weights.

RESULTS

In preliminary experiments to confirm that GBS would adhere to BEC, their adherence was compared with that of group-A streptococci, which previously have been shown to become bound to BEC (Alkan, Ofek and Beachey, 1977). There was no statistically significant difference ($p > 0.1$) between the number of these two organisms binding to BEC (table I).

TABLE I
Adherence of group-A streptococci and type-III GBS to human BEC

Bacterial cells	Mean* number of bacteria attached per epithelial cell ± 2 SEM
None (background)	2.3 $\pm$ 0.6
Type-III GBS	13.9 $\pm$ 2.5
Group-A streptococci	15.7 $\pm$ 3.2

GBS = group-B streptococci; BEC = buccal epithelial cells.

* Of count on 100 epithelial cells.

The kinetics of the binding reaction between BEC and type-III GBS, determined by incubating mixtures of GBS and BEC for 5, 15, 25, 35, 45, 60 and 90 min respectively, are shown in fig. 1. Maximum adherence was reached after 45 min with no significant increase after incubation for 90 min.

Periodate treatment of BEC

Treatment of BEC with sodium meta-periodate caused a significant decrease ($p < 0.001$) in the binding of type-III GBS to the cells (table II).

Pretreatment of bacteria

Periodate treatment of type-III GBS did not cause a decrease in binding to BEC. Mild heat treatment of bacteria, 75°C for 30 min, did not decrease the binding either but autoclaving at 121°C for 15 min reduced binding to a very low level; the difference

FIG. 1.—Effect of incubation time on adherence of type-III GBS to BEC. Each point represents the geometric mean (± 1 SE) of results from 100 buccal cells.

was statistically highly significant ($p < 0.001$). Treatment of GBS with trypsin caused a significant decrease ($p < 0.01$) in their subsequent adherence to BEC. These results are shown in table III.

Blocking of adherence with sonicate from type-III GBS

Preincubation of BEC with the sonicate preparation blocked the binding of type-III GBS in a dose-response fashion (fig. 2).

Treatment of the sonicate at 121°C for 15 min significantly reduced its ability to block bacterial binding ($p < 0.001$), but periodate oxidation of the sonicate affected its blocking capacity much less (table IV). Because there was no significant difference ($p > 0.1$) between the activities of the periodate-treated sonicate and the periodate-control sonicate, it seems unlikely that a carbohydrate on the bacterial surface is concerned in adhesion.

TABLE II
Effect of periodate treatment of BEC on subsequent adherence of type-III GBS

Cells	Mean* number of bacteria attached per epithelial cell ± 2 SEM
None (background)	10.8 ± 1.9
Periodate-treated BEC (background)	10.1 ± 1.6
BEC + GBS	19.3 ± 2.8
Periodate-treated BEC + GBS	13.0 ± 1.9

Footnotes as in table I.

TABLE III
Effect of pretreatment of type-III GBS on their adherence to BEC

Bacterial cells	Mean* number of bacteria attached per epithelial cell $\pm$ 2 SEM
None (background)	19.9 $\pm$ 3.4
GBS	26.5 $\pm$ 3.2
Periodate-treated GBS	30.0 $\pm$ 4.4
None (background)	21.4 $\pm$ 3.2
GBS	30.5 $\pm$ 3.4
Mildly heated† GBS	35.4 $\pm$ 3.5
None (background)	19.2 $\pm$ 2.6
GBS	30.5 $\pm$ 3.4
Autoclaved‡ GBS	20.0 $\pm$ 2.0
None (background)	24.5 $\pm$ 3.2
GBS	40.8 $\pm$ 5.9
Trypsinised GBS	30.8 $\pm$ 3.6

† 75 C for 30 min.

‡ 121 C for 15 min.

Other footnotes as in table I.

Inhibition of adherence by sugars

Of all the sugars tested (0.2M D-galactose, 0.2M L-fucose, 0.05M lactose, 0.2M D-glucose, 0.2M α -methyl-D-mannoside, 0.2M maltose, 0.1M N-acetyl-D-galactosamine and 0.1M N-acetyl-D-glucosamine), only N-acetyl-D-glucosamine produced a

FIG. 2.—Dose-response curve of inhibition of binding by type-III GBS sonicate. Each point represents the geometric mean ($\pm$ 1 SE) of results from 100 buccal cells. --- = background.

TABLE IV

Effect of heat and periodate oxidation on the capacity of type-III GBS sonicate to block binding of type-III GBS to BEC

Epithelial and bacterial cells	Mean* number of bacteria attached per epithelial cell $\pm$ 2 SEM
Untreated BEC (background)	2.6 ± 1.4
Untreated BEC + GBS	8.5 ± 1.6
Sonicate-treated BEC + GBS	3.2 ± 0.5
Heated† sonicate-treated BEC + GBS	6.7 ± 0.9
Periodate-oxidised sonicate-treated BEC + GBS	5.0 ± 0.8
Unoxidised control sonicate-treated BEC + GBS	4.2 ± 1.1

† 121°C for 15 min.

Other footnotes as in table I.

FIG. 3.—Dose-response curve of inhibition of binding of type-III GBS to BEC by N-acetyl-D-glucosamine. Means of three counts.

TABLE V

Effect of pretreatment of BEC with type-III GBS membrane LTA on subsequent adherence of type III-GBS

Epithelial and bacterial cells	Mean* number of bacteria attached per epithelial cell $\pm$ 2 SEM
Untreated BEC (background)	22.2 $\pm$ 2.6
LTA-treated BEC+GBS	28.9 $\pm$ 3.8
Untreated BEC+GBS	33.0 $\pm$ 3.2

LTA = lipoteichoic acid.
Other footnotes as in table I.

FIG. 4.—SDS-polyacrylamide-gel electrophoresis of sonicate preparation from type-III GBS. Lane A, molecular-weight markers; lane B, sonicate from type-III GBS.

significant decrease ($p < 0.001$) in subsequent attachment of type-III GBS to BEC. A dose-response curve is shown in fig. 3.

Pretreatment of BEC with membrane LTA

No significant difference in binding was evident between treated and untreated cells ($p > 0.05$) (table V).

Polyacrylamide-gel electrophoresis

SDS-PAGE of the sonicate preparation revealed several protein bands, with one major band of low molecular weight (c. 17 000) as shown in fig. 4.

DISCUSSION

Buccal epithelial cells have been widely used as target cells in experiments designed to study the adherence of many different bacteria to epithelial surfaces (Alkan, Ofek and Beachey, 1977; Atkinson and Trust, 1980; Craven *et al.*, 1980; Saunders and Miller, 1980; Yamazaki, Ebisu and Okada, 1981). Although a large proportion of buccal cells are non-viable, as judged by trypan-blue exclusion, several layers of dead cells are present in the buccal mucosa (Beachey, 1981). In view of this, it seems appropriate to use them as a target cell for in-vitro assays. The mechanism of attachment of group-A streptococci has been studied with the help of BEC-attachment assays (Beachey, 1975) and it was found that, in the assay described in this paper, group-A streptococci and type-III GBS were bound to BEC to the same extent.

Sodium meta-periodate cleaves the C-C bond between vicinal hydroxyl groups of sugars and when BEC were treated with periodate there was a highly significant drop in the number of GBS that adhered to the cells. Conversely, treatment of the GBS with periodate did not reduce the subsequent binding, suggesting that a sugar molecule on the surface of the buccal cells plays a role in the binding mechanism. Inhibition studies with various simple sugars indicate that N-acetyl-D-glucosamine may be concerned in this.

Mild heat treatment of the bacteria did not reduce binding but heating at 121°C for 15 min markedly reduced the degree of attachment; in both instances the bacteria were killed. Possibly the adhesin on the bacterial cell wall is a lectin, a thermostable protein not affected by a temperature of 75°C but denatured at 121°C.

Results obtained with the sonicate from type-III GBS also support the view that a protein rather than a carbohydrate on the bacterial cell surface is the relevant adhesin. Heat treatment of the sonicate greatly reduced its efficiency as a blocking agent whereas the periodate had very little, if any, effect. SDS-PAGE of the sonicate preparation indicated its heterogeneity although there was one dominant protein band of low molecular weight (c. 17 000). It now seems likely that many bacteria, including such diverse organisms as *Escherichia coli* (Ofek, Beachey and Sharon, 1978), *Eikenella corrodens* (Yamazaki *et al.*, 1981), *Aeromonas hydrophila* (Atkinson and Trust, 1980) and *Fusobacterium nucleatum* (Mongiello and Falkler, 1979) rely on lectin-type interactions for their adherence to mucosal surfaces. Work in our laboratories has also shown that *Corynebacterium parvum* may attach to macrophages through a lectin-like binding site in the cell wall of the bacterium (Bagg, Poxton and Weir, 1981).

The suggestion (Botta, 1979) that some characteristics of adhesion of GBS to human vaginal epithelial cells may be explained by a lectin-type interaction, is supported by our findings. Work recently published (Elbein *et al.*, 1981) describing the binding of GBS to influenza viral glycoproteins that have been inserted into the cell membrane of infected MDCK cells, also supports this view. In the system that we studied, in which type-III GBS attach to BEC, it seems that an N-acetyl-D-glucosamine specific lectin on the bacterial cell surface is involved in attachment of the organism to the eukaryotic cell.

Beachey (1975) has identified membrane LTA as being responsible for binding of group-A streptococci to epithelial cells. Preincubation of BEC with LTA extracted from the cytoplasmic membrane of type-III GBS failed to block significantly the attachment of GBS, so that it seems that the mechanism of their adherence differs from that of the group-A streptococci. This agrees with the finding that loss of LTA from GBS cell walls, caused by treatment with penicillin, did not inhibit adherence of the bacteria to vaginal cells (Zawaneh *et al.*, 1979).

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