A haemagglutinating adhesin of group B streptococci isolated from cases of bovine mastitis mediates adherence to HeLa cells

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Rabbit erythrocytes were agglutinated by 43·4% of group B streptococci isolated from bovines but by none isolated from humans. Haemagglutination was enhanced by cultivation of the bacteria under microaerophilic conditions. Most of the haemagglutinating strains had protein type antigen X, either alone, or in combination with polysaccharide antigens. Heat and proteolytic treatment of the bacteria destroyed the haemagglutination activity. The haemagglutinin could be solubilized from the bacterial surface by mutanolysin treatment and isolated from culture supernatant fluid by ammonium sulphate precipitation. The isolated haemagglutinin did not cause direct agglutination of erythrocytes. However, binding of the haemagglutinin to rabbit erythrocytes could be visualized by agglutination of haemagglutinin-treated erythrocytes by specific antiserum obtained by absorption. Western blotting showed that the haemagglutinin obtained from erythrocyte lysates contained an antibody-reactive band with a molecular mass of 43 kDa. Haemagglutination-positive strains adhered to HeLa cells in higher numbers than did haemagglutination-negative strains. The HeLa cell adherence of Group B streptococci was inhibited in the presence of isolated haemagglutinin or of specific antiserum against the haemagglutinin. These observations suggest that the haemagglutinating adhesins of bovine group B streptococcal isolates are directly involved in the adherence mechanisms of these organisms.

Introduction

Bacterial infections are generally initiated by the adherence of pathogenic bacteria to epithelial cells of the host organism. Adherence to host cells appears to be a multifactorial phenomenon involving specific as well as nonspecific, physicochemical interactions. Structures that are probably responsible for bacterial adhesion include fimbriae and non-fimbrial adhesins, such as lipoteichoic acid or adhesins of protein nature. Some mechanisms for adhesion and bacterial colonization of mucosal surfaces were summarized by Arp (1988). The physicochemical interactions involved in adhesion are mainly contributed by hydrophobic surface structures (Doyle & Rosenberg, 1990).

The ability of bacteria to attach to and agglutinate erythrocytes has been used *in vitro* as a model for studying interactions between bacteria and their hosts. A relation between haemagglutinating properties and the

ability of the bacteria to adhere to host cells has been observed for various bacterial species (Deslauriers & Mouton, 1992; Hoschützky *et al.*, 1989; Kanoe *et al.*, 1992; Kurl *et al.*, 1989). The present study was designed to study haemagglutinating properties of bovine isolates of group B streptococci and the possible role the haemagglutinin might play in mediating adherence of the bacteria to host cells.

Methods

Bacteria. A total of 150 streptococcal isolates of serological group B were used in this study. The cultures included the type reference strains 090 (serotype Ia), H36B (Ib), 18 RS 21 (II), 6313 (III), 3139 (IV), SS 1169 (V), A909 (Ic), 24/60 (NT/X) and 25/60 (NT/R), 83 group B streptococcal isolates from cases of bovine mastitis, and 58 group B streptococcal isolates from humans. The cultures were serotyped and further characterized as described previously (Wibawan & Lämmler, 1990, 1992). The bacteria were grown in Todd-Hewitt broth (THB, Gibco) for 18 h at 37 °C either under microaerophilic conditions in a candle jar or under aerobic conditions on a rotary shaker (60 r.p.m.).

In parallel experiments the haemagglutinating strain *Actinomyces naeshlundii* 52 was used (Lämmler *et al.*, 1987).

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Erythrocytes. Blood was collected from healthy rabbits, with 0.2 M-sodium citrate, pH 5.2, used as an anticoagulant. The erythrocytes were sedimented by centrifugation (2500 g, 10 min), washed twice with 0.15 M-NaCl and diluted in isotonic NaCl to a 2% (v/v) working suspension. In parallel experiments the erythrocytes were washed with 0.2 M-sodium acetate buffer (pH 5.2) treated with neuraminidase [100 µl erythrocyte suspension and 100 µl distilled water containing 0.1 U neuraminidase (type V neuraminidase from *Clostridium perfringens*, Sigma)] (Lämmle *et al.*, 1987), washed in 0.15 M-NaCl and diluted to the original working suspension.

Haemagglutination assay. Haemagglutination tests were carried out with 20 µl of the respective culture, adjusted photometrically (Bausch and Lomb) to 10% transmission at 620 nm (10^9 bacteria ml⁻¹ in 0.15 M-NaCl) and 20 µl of rabbit erythrocytes on microscope slides. The slides were rotated gently and within 30 s the haemagglutination was recorded as strong agglutination (+ +), agglutination (+) or no agglutination (—).

In inhibition experiments 20 µl of each bacterial suspension (10^9 bacteria ml⁻¹) was preincubated for 1 min with 20 µl of 50 mM solutions of the sugars L(+)-rhamnose, D(+)-galactose, D(+)-lactose, D(+)-glucosamine, N-acetyl-D-glucosamine or D(+)-mannose (Sigma), or with 20 µl 0.2 M-EDTA, and subsequently used in the haemagglutination assay. In parallel experiments the haemagglutination assay was performed in the presence of 20 µl 0.2 M-EDTA and 20 µl 3 mM-CaCl₂.

Treatment of bacterial cells. To study the effect of various treatments of the bacteria on haemagglutinating activities, selected isolates were heated at 50 °C for 10, 30 or 60 min, or incubated with proteolytic enzymes. The latter treatment was performed with 50 µg trypsin or pronase E (Merck) per ml of spectrophotometrically adjusted bacterial suspension [10^9 bacteria ml⁻¹ in phosphate-buffered saline (PBS, g l⁻¹: Na₂HPO₄, 12.14; NaCl, 4.35; pH 7.5)] for 1 h at 37 °C. The bacteria were subsequently washed in 0.15 M-NaCl and used in the haemagglutination assay. In parallel experiments the bacteria (10^9 bacteria ml⁻¹ in sodium acetate buffer, pH 5.2) were treated with 0.1 U neuraminidase ml⁻¹ (type V neuraminidase from *Clostridium perfringens* in distilled water) washed in 0.15 M-NaCl and used in the haemagglutination assay.

Preparation of specific antiserum against the group B streptococcal haemagglutinin. The type-specific antiserum against haemagglutination-positive group B streptococcal reference strain 24/60, with type antigen pattern NT/X, was absorbed with haemagglutination-negative group B streptococcal strain 781, also exhibiting type antigen pattern NT/X. The specificity of the absorbed antiserum was tested in immunodiffusion reactions: it did not react with group B streptococcal group or type antigen preparations. For haemagglutination inhibition tests, 20 µl of bacterial suspensions (10^9 bacteria ml⁻¹) of a haemagglutination-positive group B streptococcal culture was preincubated with 20 µl of absorbed antiserum for 5 min and subsequently used in the haemagglutination assay. For control purposes the bacteria were preincubated with monospecific antisera against haemagglutination-negative reference strains 18 RS 21 (II) and 3139 (IV), and with serum of a nonimmunized rabbit.

Isolation of haemagglutinin. Haemagglutination-positive and -negative group B streptococcal cultures were cultivated in 1 litre of THB for 18 h at 37 °C under microaerophilic conditions in a candle jar, centrifuged, washed with PBS and resuspended in 5 ml PBS containing 100 U mutanolysin (Sigma). The suspension was incubated for 1 h at 37 °C. Solubilized material was separated from cell debris by centrifugation (10000 g, 10 min, 4 °C) and the supernatant was dialysed against distilled water for 48 h at 4 °C. In parallel experiments the culture supernatant fluid of the group B streptococci was precipitated with 70% saturated ammonium sulphate (472 g l⁻¹) for 24 h at 4 °C.

The precipitate was harvested by centrifugation, resuspended in 5 ml PBS and dialysed against distilled water for 48 h at 4 °C.

Binding of solubilized and precipitated haemagglutinin to rabbit erythrocytes. This experiment was performed as described by Lindahl *et al.* (1990). Dialysed mutanolysin extract (2 ml) or ammonium sulphate precipitate (2 ml) was incubated with 1 ml of 2% (v/v) rabbit erythrocyte suspension for 1 h at room temperature. After centrifugation (1500 g, 10 min) the erythrocytes were lysed with 300 µl 2% (w/v) SDS and dialysed against distilled water for 48 h at 4 °C. The molecular mass of the haemagglutinin was estimated using an 11% SDS-polyacrylamide separating gel (Weber & Osborn, 1969) and Western blotting (Towbin *et al.*, 1979), for which the nitrocellulose membranes were developed with haemagglutinin-specific antibodies (diluted 1:25), skimmed milk [10% in 0.05 M-Tris-buffered saline (0.14 M-NaCl), pH 7.4] as blocking solution and peroxidase-conjugated anti-rabbit immunoglobulins from pigs (1:250, Dakopatts). For control purposes lysates obtained from rabbit erythrocytes which had been incubated with mutanolysin extracts of a haemagglutination-negative group B streptococcal culture and serum from a non-immunized rabbit were used.

Adherence to HeLa cells. HeLa S3 cells were grown for 24 h at 37 °C in minimum essential medium (MEM, Sigma) containing 10% (v/v) foetal calf serum (Gibco), in cell culture dishes containing glass slides (10 mm × 20 mm) to form a nonconfluent monolayer. The glass slides were then submerged in Petri dishes containing 4 ml Hanks' balanced salt solution (HBSS, Sigma) and subjected to the adherence assay. For this assay the bacteria were grown for 18 h at 37 °C in THB under microaerophilic conditions in a candle jar, centrifuged, washed with HBSS, adjusted spectrophotometrically to 10^9 bacteria ml⁻¹ and stained with fluorescein isothiocyanate (FITC, 1 mg ml⁻¹ in HBSS) for 1 h at room temperature at a final concentration of 0.25 mg FITC per ml of bacterial suspension. The bacteria were washed twice with HBSS and stored at 4 °C until use. To study the adherence properties of the bacteria, 100 µl of the bacterial suspension was added to the HeLa-cell-coated slides in 2 ml HBSS and incubated for 1 h at 37 °C. The slides were then gently washed with HBSS containing 0.05% Tween 20. The number of adhering bacteria was counted with a fluorescence microscope.

In parallel experiments the adherence tests were performed with bacteria pretreated with specific antiserum against haemagglutinin or serum of a nonimmunized rabbit (10^9 bacteria ml⁻¹, 50 µl serum, 1 h, 37 °C). In addition, the HeLa cells were used after pretreatment with 50 µl isolated haemagglutinin. The haemagglutinin was recovered from SDS lysates of rabbit erythrocytes pretreated with mutanolysin extracts or ammonium sulphate precipitate from culture supernatant fluid of a haemagglutination-positive group B streptococcal cultures as described. Lysates from rabbit erythrocytes were used as control.

Results

Haemagglutination

Of the 83 group B streptococcal strains of bovine origin used in this study, 36 (43.4%) agglutinated rabbit erythrocytes. The reference strain 24/60 with type antigen pattern NT/X also agglutinated rabbit erythrocytes. All other reference strains and all strains isolated from humans were non-haemagglutinating. Of the 36 haemagglutination-positive strains, 30 had type antigen X, either alone or in combination with type antigen II or IV, one had the type antigen pattern II/c and five were non-typable. Twice as many bovine isolates

Table 1. Distribution of haemagglutination reactions with rabbit erythrocytes among group B streptococcal isolates of various serotypes grown under microaerophilic and aerobic conditions

B streptococcal strains	No. of strains	No. showing haemagglutination*					
		Microaerophilic			Aerobic		
		++	+	-	++	+	-
Bovine serotypes							
II	8	0	0	8	0	0	8
II/c	8	0	1	7	0	1	7
II/X	4	3	1	0	0	0	4
IV	14	0	0	14	0	0	14
IV/c	9	0	0	9	0	0	9
IV/X	18	18	0	0	1	11	6
NT/X	10	5	3	2	0	4	6
NT	12	1	4	7	0	0	12
Human isolates							
Various serotypes	58	0	0	58	0	0	58

* Haemagglutination activity after growth under the conditions shown: ++, strong haemagglutination; +, haemagglutination; -, no haemagglutination.

were haemagglutinating after growth under microaerophilic conditions as under aerobic conditions (Table 1).

Heat treatment of the bacteria for 30 min at 50 °C abolished the haemagglutination activities, as did trypsin and pronase treatment, indicating that the haemagglutinin was proteinaceous. Treatment of the rabbit erythrocytes or the haemagglutinating bacteria with neuraminidase had no effect on haemagglutination. Selected haemagglutination-negative group B streptococci from bovines and humans were still negative after neuraminidase treatment. In contrast, the haemagglutination reaction of *Actinomyces naeslundii* was enhanced after neuraminidase treatment of the rabbit erythrocytes.

The haemagglutination reactions of the group B streptococci and of *A. naeslundii* were abolished by the addition of EDTA. The addition of CaCl₂ to EDTA-pretreated bacteria restored the haemagglutination reactions. The presence of L(+)rhamnose, D(+)galactose, D(+)lactose, D(+)glucosamine, N-acetyl-D-glucosamine and D(+)mannose had no effect on the haemagglutination reactions of the group B streptococci. The haemagglutination of *A. naeslundii* was inhibited in the presence of D(+)lactose and D(+)galactose.

The surface proteins responsible for the haemagglutination reactions could be solubilized by mutanolysin treatment of the bacteria or be precipitated from culture supernatant by ammonium sulphate. Rabbit erythrocytes were incubated with both haemagglutinin preparations and subsequently lysed by the addition of SDS.

Fig. 1. Western blot analysis of haemagglutinin preparations from mutanolysin extract (A) and ammonium sulphate precipitate of culture supernatant fluid (B) from group B streptococcal strain 395/2. The nitrocellulose membranes were developed with haemagglutinin-specific antiserum.

Both the mutanolysin-released and the ammonium-sulphate-precipitated lysates reacted specifically with the haemagglutinin-specific antiserum in immunodiffusion reactions. No precipitation reaction was observed with serum of a nonimmunized rabbit or with antisera against non-haemagglutinating strains. Incubation of rabbit erythrocytes with crude haemagglutinin preparations or with both lysates did not cause direct haemagglutination. Haemagglutination was observed after the addition to haemagglutinin-treated rabbit erythrocytes of specific antiserum prepared against the haemagglutinin (see Methods), thereby demonstrating binding to the erythrocytes. Control serum of a non-immunized rabbit or monospecific antisera against haemagglutination-negative group B streptococci had no effect. Preparations obtained from a haemagglutination-negative group B streptococcal culture were negative in all these tests.

SDS-PAGE and Western blot analysis of the lysates revealed a reactive band with a molecular mass of 43 kDa. The Western blot was developed with haemagglutinin-specific antiserum. Controls with serum of a non-immunized rabbit or lysates from erythrocytes obtained after incubation of the erythrocytes with mutanolysin extracts of a haemagglutination-negative group B streptococcus showed no comparable reaction (Fig. 1).

Table 2. Adherence of selected haemagglutination-positive group B streptococcal isolates of bovine origin to HeLa cells before and after various treatments

Mean values $\pm$ SD (four separate experiments) of bacterial adherence to HeLa cells are shown, with the range of results in parentheses. ND, Not determined.

Potential inhibitor in adherence assay*	No. of adherent bacteria per HeLa cell			
	Haemagglutinating		Non-haemagglutinating	
	395/2	747	781	G28
Control	115.90 $\pm$ 2.65 (112.10–118.25)	108.78 $\pm$ 6.31 (99.35–112.85)	6.45 $\pm$ 1.05 (5.10–7.35)	4.89 $\pm$ 0.63 (4.05–5.40)
Haem-M	8.53 $\pm$ 2.06 (6.55–10.95)	8.35 $\pm$ 3.25 (5.10–12.60)	ND	ND
Haem-S	13.33 $\pm$ 5.97 (6.10–16.95)	7.50 $\pm$ 2.40 (3.95–9.15)	ND	ND
Haem-As	7.83 $\pm$ 3.35 (4.15–11.40)	9.08 $\pm$ 3.42 (5.70–13.35)	ND	ND
NS	111.03 $\pm$ 3.11 (108.45–109.55)	112.20 $\pm$ 16.11 (89.60–126.70)	ND	ND

* Haem-M, 50 μ l haemagglutinin preparation from mutanolysin extracts; Haem-S, 50 μ l haemagglutinin from ammonium sulphate precipitate of culture supernatant fluid; Haem-As, 100 μ l specific antiserum against haemagglutinin; NS, 100 μ l serum of a non-immunized rabbit.

Adherence to HeLa cells

Selected representative haemagglutination-positive and haemagglutination-negative group B streptococcal strains (395/2, 747 and 781, G28, respectively) were used in HeLa cell adherence tests. The haemagglutination-positive group B streptococci adhered to HeLa cells with more than 100 bacteria per HeLa cell, but fewer than 10 bacteria adhered per HeLa cell for the haemagglutination-negative strains (Table 2).

The adherence of haemagglutination-positive group B streptococci to HeLa cells was greatly reduced in the presence of haemagglutinin preparations obtained from lysates of erythrocytes incubated with mutanolysin extracts or from lysates of erythrocytes incubated with ammonium sulphate precipitates of culture supernatant fluid. No inhibition of adherence was observed with lysates from control erythrocytes. A comparable inhibition of HeLa cell adherence was observed in the presence of specific antiserum against haemagglutinin. Serum of a non-immunized rabbit had no effect (Table 2).

Discussion

The attachment of bacteria to erythrocytes, leading to haemagglutination, has been described as a common property of streptococci of serological group G, but less pronounced among those of serological groups C and D. Among 130 group B streptococci examined by Kurl *et al.*

(1989), only one culture displayed haemagglutinating activities. The low number of haemagglutination-positive cultures observed might have been related to the origin of the group B streptococci. In the present study 36 of 83 bovine group B streptococci isolates agglutinated rabbit erythrocytes. In contrast, none of the group B streptococcal isolates from humans did so.

Most of the haemagglutination-positive group B streptococci, including type reference strain 24/60, had type antigen X, either alone, or in combination with polysaccharide antigens. However, the haemagglutinating properties seemed not to be directly related to the occurrence of type antigen X, because haemagglutinating activities were also observed among non-typable strains and with a group B streptococcal strain with type antigen pattern II/c. Compared to the results of previous studies (Wibawan & Lämmle, 1992; Wibawan *et al.*, 1992) it was of interest that all the haemagglutination-positive group B streptococci displayed hydrophobic surface properties. However, not all strains with hydrophobic surface structures were haemagglutination-positive. In addition the haemagglutinating activities were markedly influenced by the cultivation conditions. Under microaerophilic conditions the number of haemagglutination-positive bacteria increased. Cultivation under microaerophilic conditions has also been shown to enhance the microcapsule production of group B streptococci (Wibawan & Lämmle, 1992). However, the haemagglutinating activities seemed not to be influenced by the neuraminic acid part of the group B streptococcal microcapsule: the removal of neuraminic

acid by neuraminidase treatment did not alter the haemagglutination reactions and did not yield a positive haemagglutination reaction with haemagglutination-negative strains. In contrast, a masking effect of neuraminic acid has been observed for hydrophobic surface proteins and fibrinogen-binding structures (Chhatwal *et al.*, 1984; Wibawan & Lämmler, 1991, 1992).

In addition, treatment of the rabbit erythrocytes with neuraminidase did not change the haemagglutination activities. This corresponded to the findings of Kurl *et al.* (1989). The haemagglutinin of the group B streptococci was susceptible to heat and proteolytic treatment of the bacteria, indicating its protein nature. As already described for *A. naeslundii* (Lämmler *et al.*, 1987) the haemagglutination of group B streptococci could be inhibited by EDTA, showing the Ca^{2+} -dependency of the reaction. Comparable to the findings of Kurl *et al.* (1989), none of the sugars used in this study inhibited group B streptococcal haemagglutination reactions.

The haemagglutinin of group B streptococci could be solubilized from the bacterial surface by mutanolysin treatment or isolated from culture supernatant fluid by ammonium sulphate precipitation. This indicates that the haemagglutinin is not only cell bound but also released into the culture medium. Neither haemagglutinin preparation caused a direct agglutination of the rabbit erythrocytes. However, binding of both agglutinins to the erythrocytes could be visualized by an agglutination reaction with a haemagglutinin-specific antiserum. Both types of haemagglutinin preparations could be released from the erythrocyte surface by SDS treatment of the erythrocytes. After SDS-PAGE and Western blotting a single band with a molecular mass of 43 kDa was identified by using absorbed antiserum.

Both haemagglutinin preparations seemed to mediate the adherence of the bacteria to HeLa cells. Haemagglutination-positive group B streptococci adhered in higher numbers to HeLa cells than did haemagglutination-negative strains. In addition, haemagglutinin preparations and specific antiserum against haemagglutinins almost completely inhibited the interaction between the bacteria and the HeLa cells.

The initial step in bacterial infection involves the contact of the bacterial surface with host cells. For group B streptococci, lipoteichoic-acid-mediated adherence and adherence without lipoteichoic acid involvement has been reported (Miyazaki *et al.*, 1988; Nealon & Mattingly, 1984; Wibawan *et al.*, 1992). The latter included hydrophobic interactions. According to Nealon & Mattingly (1984) hydrophobic interactions are the first step in binding to embryonic and foetal cells, followed by a secondary attachment involving lipoteichoic acid. The adherence to epithelial cells may involve only one

process, that is hydrophobic interaction. Bramley & Hogben (1982) reported a protein-mediated adherence of group B streptococci.

The results of the present study show that at least for bovine group B streptococcal isolates a haemagglutinating adhesin seems to be involved in adherence. This haemagglutinin might be one part of multiple group B streptococcal adhesins and contribute to the ability of these organisms to adhere to mucosal surfaces. The occurrence of multiple adhesins in streptococcal species has been reviewed by Hasty *et al.* (1992). In addition, the present results contribute to previous findings that group B streptococci could be divided into hydrophobic, haemagglutinating, cell-adherent strains and hydrophilic, non-haemagglutinating, encapsulated strains. The latter are less readily phagocytosed by polymorphonuclear leucocytes (Wibawan *et al.*, 1992). As described by Sellin *et al.* (1992) both properties might occur in phase variants of individual group B streptococcal strains.

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