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Role of Adherence in the Pathogenesis of Neonatal Group B Streptococcal Infection

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The ability of group B streptococci to attach to buccal epithelial cells from adult volunteers, healthy neonates, and infants with invasive group B streptococcal infection was assessed by using ^{3}H-labeled bacteria incubated at a bacteria-to-cells ratio of 1,000:1. Type III group B streptococcal clinical isolates adhered significantly better to the epithelial cells of healthy neonates than to those of adults (mean bacteria per cell of 31 versus 7, respectively; $P < 0.005$). In contrast, no statistically significant differences in adherence of type Ia or type II strains to cells of neonates and adults were noted. The adherence of strains isolated from 15 infants with invasive group B streptococcal infection was significantly greater to the cells of infected infants than to those of age-matched controls (mean bacteria per cell of 39 versus 18, respectively; $P < 0.005$). In contrast, no significant difference was noted in the adherence of a usually adherent type Ia strain and a nonadherent type III strain to the cells of infected infants compared with control infants. These results indicate that the serotype of group B streptococci with the greatest virulence for neonates (type III) adheres better to neonatal than to adult epithelial cells. Infants who develop invasive infection may have an increased number of epithelial cell surface receptor sites for attachment of group B streptococci, the bacteria may elaborate products which unmask receptor sites, or both.

Adherence of bacteria to tissue surfaces is a potentially initial event in the pathogenesis of bacterial infections (5, 6, 8, 11, 13, 17). In those infections in which bacteremia ensues, a stepwise process might be envisioned in which organisms first adhere to host epithelial cell surfaces, replicate, and eventually penetrate these cell barriers. Penetration may be effected by extracellular products or by properties of bacterial cells themselves.

Since 1970, group B streptococci have been frequent isolates from neonates and young infants with septicemia and meningitis. Attack rates have varied from 1.6 to 3.7 per 1,000 live births (1, 2, 16). Although certain factors critical to the pathogenesis of these infections have been delineated, little information regarding the adherence of these microorganisms to human epithelial cells (7, 14, 25) has been reported and only one study has utilized neonatal cells (14).

The purpose of the studies reported here was threefold: (i) to determine the adherence of a variety of clinical group B streptococcal isolates to the buccal epithelial cells (BEC) of healthy adults and neonates, (ii) to define patterns of adherence by strain serotype, source of isolate, and host age, and (iii) to determine a possible role of adherence in the pathogenesis of group B streptococcal infection. The latter was approached by comparing the adherence of several group B streptococcal strains to the BEC from infants with invasive group B streptococcal infection with adherence to the cells from a group of control infants.

MATERIALS AND METHODS

Bacterial strains. Twenty-seven clinical group B streptococcal isolates (5 type Ia, 3 type II, 17 type III, and 2 nontypable) were evaluated. Twenty-five of these strains were isolated from the blood (11) or cerebrospinal fluid (14) of infants with group B streptococcal infections of various clinical severities. An additional two nontypable strains (kindly supplied by Patricia Ferrieri, University of Minnesota, Minneapolis, Minn.) were genital isolates from asymptomatically colonized adults. Prototype strains Ia (090) and II (18RS21) that had been passed in mice to increase capsule size and virulence by the late Rebecca Lancefield (The Rockefeller University, New York) were also evaluated. In addition, a less encapsulated, less mouse-virulent variant of the prototype Ia strain was studied (4). All isolates were stored at −20°C in 1-ml samples of Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.) before their use.

Study populations. The BEC of 9 adult laboratory workers and 26 healthy term neonates were studied.

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The neonates ranged in age from 1 to 6 days (mean 2.2 days) when evaluated. None of these infants or adult volunteers was receiving antibiotics. Each infant had a throat culture obtained on the day of study to determine group B streptococcal colonization status (3).

Fifteen infants with group B streptococcal bacteremia or meningitis were also evaluated. BEC from these infants were compared on the same day with those from a group of 15 age-matched control infants. The group B streptococci-infected infants were all born after term gestations. Five had early onset and 10 had late onset illness. Nine patients had meningitis, and six had bacteremia without meningitis. Thirteen infections were caused by type III and two by type Ia group B streptococci. All infants were studied several days after the diagnosis was established and were receiving antimicrobial therapy, usually ampicillin or penicillin. Of the 15 control infants, six were hospitalized to rule out bacterial sepsis but had negative cultures, two had pyloric stenosis, and one each had *Escherichia coli* meningitis, diarrhea, congenital heart disease, respiratory distress of undetermined etiology, chlamydial conjunctivitis, or neonatal drug withdrawal. One infant was completely well. Eight of the control infants were receiving antibiotic therapy at the time of study.

Adherence assay. An adherence assay employing [^{3}H]thymidine-labeled group B streptococci and BEC was modified after that of Sugarman and Donta (21). Group B streptococcal strains were inoculated onto 5% sheep blood agar plates and incubated overnight at 37°C, and stationary-phase cultures were prepared by incubating bacteria with 25 μCi of [*methyl*-^{3}H]thymidine (New England Nuclear Corp., Boston, Mass.) in 5 ml of Todd-Hewitt broth at 37°C for an additional 16 h. The bacterial suspensions were then centrifuged, washed three times with Dulbecco phosphate-buffered saline (PBS; M.A. Bioproducts, Walkersville, Md.), pH 7.4, and suspended in PBS to an optical density of 0.16 at 540 nm (Spectronic 20, Bausch & Lomb Inc., Rochester, N.Y.). Such suspensions contained ~10^8 CFU/ml; optical density and colony counts of the inoculum were assessed on each test day. BEC were collected by gently scraping the buccal mucosa with a tongue depressor and placing the cells in 10 ml of PBS. Cells were washed with PBS and brought up to a final concentration of ~10^5 cells per ml. The percentage of viable BEC was determined with a trypan blue exclusion stain (Eastman Kodak Co., Rochester, N.Y.).

For each assay, equal volumes (1 ml) of bacteria and BEC were prepared in duplicate (ratio of bacteria to BEC, ~1,000:1). Similar suspensions containing bacteria and PBS were prepared as a control for the autoagglutination of bacteria and to account for potential trapping of bacteria on filters. Suspensions were rotated at 37°C for 1 h, filtered through 12-μm polycarbonate filters (Nuclepore Corp., Pleasanton, Calif.), and washed four times with PBS. Duplicate 1-ml volumes of the bacterial suspensions alone were filtered through 0.2-μm polycarbonate filters. Filters were placed in glass scintillation vials, 1.5 ml of a 90% solution of NCS tissue solubilizer (Amersham Corp., Arlington Heights, Ill.) was added to each vial, and vials were incubated at 22°C for 20 min. Then 10 ml of OCS (Amersham) was added to each vial and, 1 h later, radioactive counts (counts per minute) were detected in a liquid scintillation counter (Tri-Carb Scintillation Spectrometer-model 3003; Packard Instrument Co., Downers Grove, Ill.).

Adherence was calculated in the manner shown below and expressed as the number of adherent bacteria per BEC.

$$\begin{gathered}\text{(group B streptococci CFU/cpm)} \\ \text{[filter cpm (group B streptococci and BEC) } - \\ \text{filter cpm (group B streptococci and PBS] } = \\ \text{no. of adherent group B streptococci per filter} \end{gathered} \quad (1)$$

$$\begin{gathered}\text{no. of adherent group B streptococci per filter/} \\ \text{no. of BEC per filter } = \\ \text{no. of group B streptococci per BEC} \end{gathered} \quad (2)$$

To assess the influence of antibiotic exposure on adherence, experiments were performed which tested the effect of preincubation of BEC of three healthy adults with solutions of PBS containing 20 or 2 μg of ampicillin per ml or no ampicillin (PBS alone) on the adherence of four group B streptococcal isolates. Cells were incubated with ampicillin (or PBS alone) for 30 min at 22°C, washed three times with PBS, and then used in the assay previously described.

To determine the effect of epithelial cell viability on adherence, two group B streptococcal strains were adhered to standard and heat-treated (65°C for 30 min) BEC of two adults and two healthy neonates.

Statistical analysis of data. All data were analyzed by *t* tests (paired or independent) or chi-square tests (20).

RESULTS

A linear relationship between the ratio of bacteria to BEC (range tested, 290 to 11,670:1) and adherence was observed. Therefore, a ratio of 1,000:1 was selected for use in the radiolabel assay and was noted to provide highly reproducible results. The latter was shown by testing a single strain with the BEC from two adults divided into eight equal portions and tested on a single day. The mean ± the standard deviation of adherence of this isolate to cells from these two volunteers was 62 ± 14 and 130 ± 21 bacteria per cell, respectively.

Adherence of group B streptococci to BEC of adults and healthy neonates. The adherence of several clinical group B streptococcal isolates to the BEC of healthy adults and neonates is graphically summarized in Fig. 1. The mean adherence of seven type III isolates to neonatal cells was significantly greater than that to adult cells (31 versus 7 bacteria per cell; $P < 0.005$, independent *t* test). No significant differences in adherence of four type Ia and three type II strains to cells from neonates and adults were detected. Interestingly, the most adherent group B streptococcal isolates to adult cells were nontypable strains, which adhered significantly less well to neonatal cells (mean 47 versus 122 bacteria per cell, respectively; $P < 0.05$, independent *t* test).

Most BEC employed in these experiments

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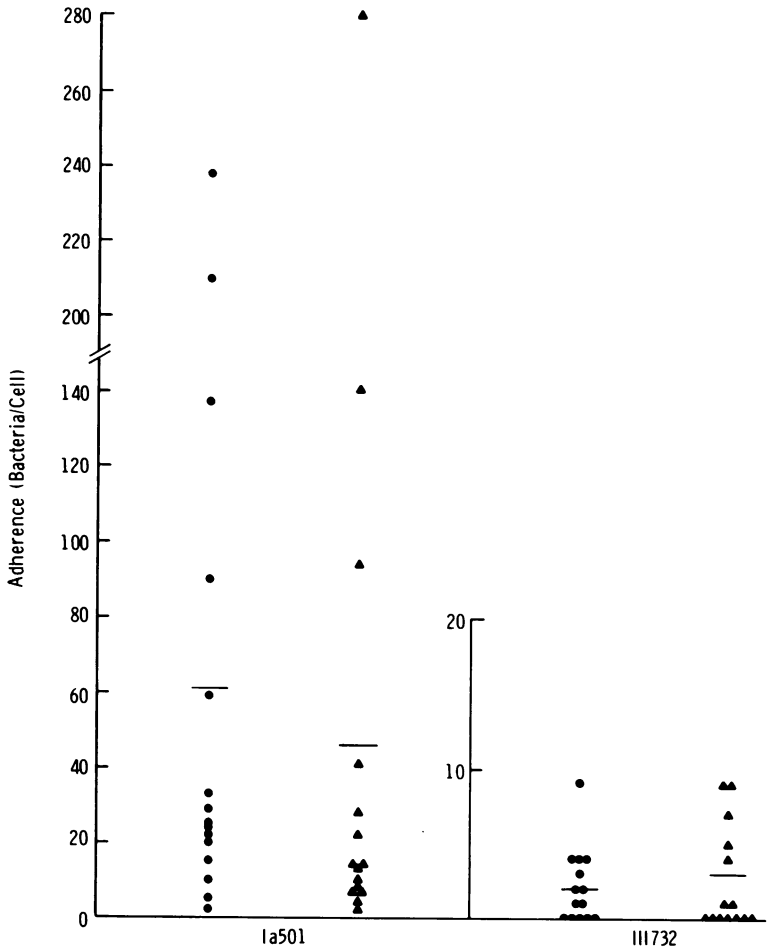

FIG. 1. Comparison of the aherence of group B streptococcal isolates representing various serotypes to adult (●) and neonatal (▲) BEC. The solid horizontal lines signify the means for each serotype tested with BEC from multiple individuals.

were nonviable as determined by trypan blue exclusion staining. Heating to 65°C caused all cells to be nonviable. Cells from neonates had greater viability than those from adults (mean ± the standard deviation, 10.4 ± 7% compared to 3.2 ± 2.3%, respectively; $P < 0.001$, t test). Therefore, the potential effect of epithelial cell viability on adherence was evaluated. When BEC from two healthy neonates and two adult volunteers were heated at 65°C for 30 min, no difference in the attachment of two group B streptococcal isolates was noted when compared with unheated cells.

The Lancefield prototype strains Ia (090) and II (18RS21), passed in mice, adhered to neither adult nor infant BEC. However, the less encapsulated variant of strain 090 did adhere to both adult and neonatal cells (10 ± 11 and 12 ± 6 bacteria per cell, respectively).

Adherence of group B streptococci to BEC from infants with invasive infection versus controls. Strains investigated included the infecting isolates from each infant with group B streptococcal bacteremia or meningitis, a type III clinical isolate (732) which adhered poorly to the BEC from adults and healthy neonates, and a type Ia clinical isolate (501) which adhered well to both of these groups. The adherence of the infecting isolates from infants with invasive group B streptococcal infection to their cells compared with cells from age-matched controls is shown in Fig. 2. The infecting isolates adhered better to cells from 12 of the 15 infected patients than they did to cells from controls. The mean ± the standard deviation of the adherence of the infecting strains to the infected infants' cells (39 bacteria per cell) was significantly greater than their adherence to the controls' BEC (18 bacteria per cell; $P < 0.025$, paired t test). When adherence was categorized (low, <10 bacteria

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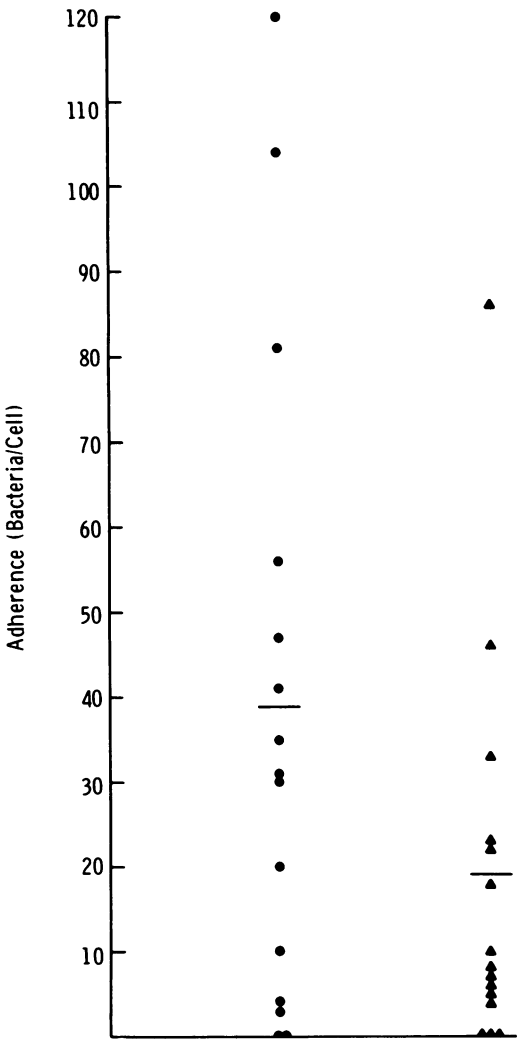

FIG. 2. Adherence of infecting group B streptococcal isolates to BEC of infants with invasive group B streptococcal infection (●) and age-matched controls (▲). The solid horizontal lines signify the means.

per cell; moderate, 10 to 25 bacteria per cell; high, >25 bacteria per cell), 60% of group B streptococci-infected infant BEC but only 20% of control infant BEC had high adherence. In contrast, the cells of 53% of the controls but only 27% of infected infants adhered the infecting strains poorly ($P < 0.05$, chi-square test). There was no significant difference between the adherence of the 13 type III infecting isolates to the control infant cells and the adherence of the similar (but not identical) group of type III clinical isolates described previously to adult cells. Neither age of onset nor focus of infection significantly influenced the adherence of the infecting isolates.

Figure 3 summarizes the attachment of a usually adherent type Ia strain, 501, and a nonadherent type III strain, 732, to the BEC of infected and control infants. The mean adherence of the type Ia strain to the BEC of group B streptococci-infected infants was not significantly greater than that to the cells of controls (61 versus 47 bacteria per cell, respectively). The type III strain, 732, adhered poorly to the BEC from both group B streptococci-infected and control infants.

Eight control infants and all infected infants were receiving antimicrobial therapy at the time adherence was assessed. Therefore, the effect of antibiotic therapy on adherence was evaluated. The adherence of the group B streptococci-infected infant isolates, strain Ia 501 and strain III 732, to the BEC of control infants receiving antibiotics was compared with adherence to the cells of infants not receiving antimicrobial therapy. As shown in Table 1, there was a tendency for the infecting isolates and strain Ia 501 to adhere better to the BEC of infants who were not receiving antibiotics than to the BEC of those infants receiving antibiotics. The differences, however, were not statistically significant. Similarly the mean ± the standard deviation of adherence of four group B streptococcal strains to the BEC from three adults was similar after preincubation of cells with 20 (163 ± 127 bacteria per cell) and 2 μg of ampicillin (165 ± 91 bacteria per cell) per ml compared with PBS preincubation (171 ± 120 bacteria per cell).

DISCUSSION

These studies were undertaken in an effort to determine whether adherence of group B streptococci to epithelial cells might play a role in the pathogenesis of infections caused by these organisms. The investigation was directed first at testing the hypothesis that the ability of oral epithelial cells to bind group B streptococci might be greater for neonates than for adults. Clinical isolates of type III group B streptococci adhered significantly better to the BEC of healthy neonates less than 1 week of age than to those of adults. Viewed differently, type III strains adhered very poorly to each of the BEC from seven adults tested. The differences observed could not be accounted for by the increased viability of BEC collected from neonates compared with those collected from adults, since additional experiments with type III strains demonstrated that epithelial cell viability did not significantly influence adherence. No statistically significant differences between the adherences of type Ia and type II strains were detected. However, nontypable strains adhered very well to adult cells, and the mean adherence was significantly greater when com-

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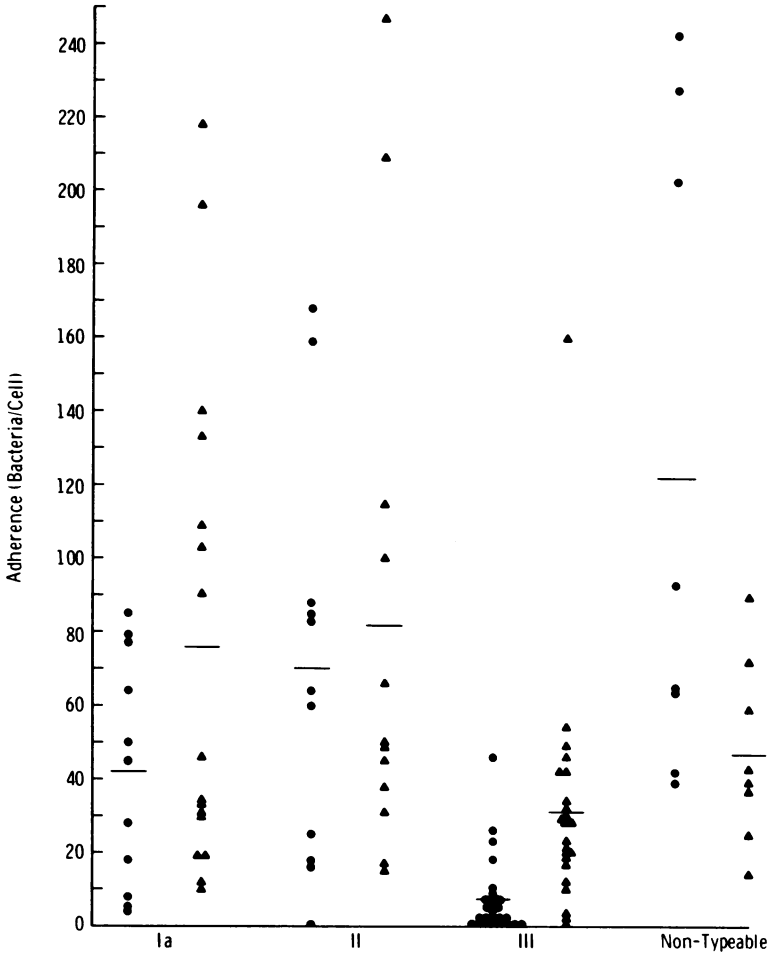

FIG. 3. Adherence of group B streptococcal isolates Ia 501 and III 732 to the BEC of infants with invasive group B streptococcal infection (●) and age-matched controls (▲). The solid horizontal lines signify the means.

pared with that for neonatal cells ($P < 0.05$).

Type III strains of group B streptococci appear to possess the greatest virulence for the neonate, since they have been reported to account for over 60% of isolates from infants with invasive group B streptococcal infection but only 30% of those from asymptomatically colonized neonates (1, 2, 24). This disparity has not been noted for strains representing the other four serotypes. Of interest, however, whereas type II strains are associated with nearly one-fourth of the bacteremic group B streptococcal infections in neonates (24), they are virtually never associated with concomitant meningeal invasion, but are the most frequent isolates from adults with group B streptococcal meningitis (12, 24). Thus, the serotype associated with the greatest virulence properties for the neonate, type III, is more adherent to the oral epithelial cells of the susceptible neonatal host than to those of the relatively nonsusceptible adult host. This finding is similar to those of other investigators who have noted a positive correlation between the known predisposition of susceptible hosts to develop infections caused by specific microorganisms and the ability of their tissues to

TABLE 1. Influence of antibiotic therapy on the adherence of group B streptococcal isolates to BEC of control infants

Strains	Adherence (mean ± SD bacteria per cell) to controls[a]	
	Antibiotics	No. antibiotics
Infecting isolates	13 ± 6	24 ± 30
Ia 501	24 ± 31	71 ± 104
III 732	3 ± 4	3 ± 4

[a] No statistically significant differences were noted for cells incubated with or without antibiotics.

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bind these pathogens (7, 11, 13, 19, 22).

The observation that nontypable strains, rarely associated with invasive infection in the human host, adhered well to both neonatal and adult BEC, but particularly to adult cells, serves to emphasize that adherence is not the sole requirement for the development of infectious diseases. Bacterial virulence may be related to other factors, including chemotaxis, elaboration of toxins, penetration of mucosal surface barriers, and resistance to host defenses (13).

Experiments evaluating the adherence of the highly encapsulated, mouse-passed Lancefield prototype strains Ia (090) and II (18RS21) and the less encapsulated laboratory-adapted 090 variant to neonatal and adult BEC were done in a preliminary effort to identify the adhesin mediating the attachment of group B streptococci to BEC. Neither prototype strain adhered appreciably to neonatal or adult cells, suggesting that the adhesin involved in the adherence of type Ia and II group B streptococcal strains to BEC may be masked by the presence of a large capsule. Further support for this concept was found in the enhanced adherence of the type Ia variant, which was laboratory adapted for less encapsulation (4). No additional testing was done, however, to identify the adhesins of type Ia or II strains or the other serotypes. Previously, lipoteichoic acid has been identified as the substance mediating the binding of group A streptococci to human oral mucosal cells (6, 15). However, a recent report suggests that type III group B streptococci have no cell wall teichoic acid (9). Other potential adhesins for group B streptococci have not been investigated, but recent studies of group A streptococci have suggested that M protein antigens and opacity factor mediate attachment (10, 23). Further studies will be required to delineate the adhesin or adhesins for type III as well as the other serotypes of group B streptococci.

Another major finding from this study was that the infecting isolates from infants with invasive group B streptococcal disease adhered significantly better to the BEC of these infants than to those of a group of age-matched controls. No such difference was observed when a usually adherent type Ia (501) and a nonadherent type III (732) clinical isolate were tested. The observation of enhanced adherence of the infecting isolates to cells of infected infants is similar to the finding of Selinger et al. (18), who determined that a strain of group A *Streptococcus* isolated from a patient with rheumatic fever adhered more avidly to pharyngeal cells of nine patients with rheumatic heart disease than to cells of controls. It is possible that neonates at risk for invasive group B streptococcal infection may have an increased number of BEC receptor sites for certain strains of group B streptococci or that GBS may elaborate products which unmask these receptors (or both), thereby promoting infection.

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