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Factors Influencing Adherence of Group B Streptococci to Human Vaginal Epithelial Cells

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Factors affecting the adherence of group B streptococci to human vaginal epithelial cells in vitro were examined. Maximal adherence was achieved within 15 min of incubation of bacteria with epithelial cells. Adherence was temperature and pH dependent; maximal adherence occurred at 37°C and pH 5.5. Killing of streptococci with ultraviolet light or penicillin did not affect adherence. Similarly, adherence was not altered by preincubating epithelial cells at 65°C for 30 min. Thus neither bacterial nor epithelial cell viability appears to be a prerequisite for adherence. Preincubation of streptococci at 65°C for 30 min resulted in a marked decrease in adherence, whereas preincubation of group B streptococci with neuraminidase was associated with a significant increase in adherence. The adherence of strains belonging to five different group B streptococcal serotypes was not altered by group-specific or type-specific rabbit antisera. These findings suggest that the site for adherence on the bacterial cell wall is heat sensitive and is masked by sialic acid, but is not related to either group-specific or type-specific antigens.

Colonization of the vaginal tract has been implicated as the major cause for transmission of group B streptococci (GBS) to newborn infants (3, 4, 5, 13). Previous studies by Franciosi et al. (13), as well as our recent studies (2), have revealed that the rate of anorectal colonization in pregnant women was higher than that of vaginal colonization. This finding, together with data reported by Kexel and Beck (16) showing that the rate of colonization of the vaginal tract is lower when cultures are obtained from the upper vaginal canal as compared to the external vaginal orifice, suggest that vaginal colonization may be secondary to contamination from the anorectal site (2, 25).

The present study was undertaken to examine local factors that influence vaginal colonization by GBS. Because adherence of bacteria to epithelial cell surfaces is an important step in colonization of the mucous membranes by microorganisms (11, 26), factors influencing adherence of GBS to vaginal epithelial cells in vitro were investigated. Our data indicate that adherence of GBS to vaginal epithelial cells occurred rapidly and was affected by the temperature of incubation and the pH of the medium. Bacterial viability and epithelial cell viability do not appear to be a prerequisite for adherence.

MATERIALS AND METHODS

Collection and preparation of vaginal epithelial cells. Vaginal epithelial cells were donated by normally menstruating women and by a pregnant woman during the third trimester of pregnancy. These studies were performed after an informed consent was signed by the subjects involved. The cells were procured by placing a sterile cotton-tipped swab in the vagina and swabbing gently the upper lateral third of the vaginal wall. This procedure provided recently exfoliated epithelial cells that were free from cells of cervical origin. The epithelial cells were immediately suspended in minimal essential medium plus Earle base (MEM; GIBCO, Grand Island, N.Y.), buffered to a final pH of 7.2 with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), by rotating the swab inside a tube containing 10 ml of the medium. All experiments were started within 1 h after collection of the cells.

The epithelial cells were washed twice by centrifugation at 35 to 45 × *g* for 5 min. The pelleted cells were suspended in 10 ml of fresh MEM and further freed of secretions and unattached bacteria by rinsing with 5 volumes of fresh MEM through an 8-μm membrane filter (Millipore Corp., Bedford, Mass.) mounted on a syringe, using forward and reverse pressure. The washed epithelial cells that collected on the surface of the filter were suspended in 10 ml of fresh MEM. The concentration of the washed epithelial cells was determined by using a hemacytometer. The cells were then

adjusted to a final concentration of 2×10^4 cells per ml in MEM.

GBS strains. GBS were isolated from pregnant women as described in a previous study (2). Beta-hemolytic colonies were picked from blood agar plates, identified preliminarily as GBS by hippurate hydrolysis (10), then identified serologically as to group-specific and serotype reactivity by the Lancefield capillary precipitin technique (17). Group-specific antiserum and type-specific antisera were kindly supplied by Rebecca Lancefield, Rockefeller University, New York. Bacterial strains were maintained either lyophilized or frozen at −70°C until used.

Preparation of GBS for adherence studies. (i) Measurement of bacterial growth. Quantitative determination of the growth of GBS was based on the turbidity of the cultures. An overnight growth (18 h) of GBS was diluted several-fold, and the optical density of each dilution at 650 nm on a Coleman Junior spectrophotometer was correlated with the number of colony-forming units (CFU) per milliliter as determined by the pour-plate technique. A linear relation between the optical density of the bacterial suspension and number of CFU found in that suspension was obtained. The average of two determinations was plotted and used as the standard curve for experimental quantitative determination of the growth of GBS.

(ii) The growth curve of GBS. Growth curves were determined for five strains of GBS representing the five serotypes. The growth curves obtained for the five strains were almost identical. The lag phase lasted less than 2 h and was followed by a logarithmic phase of growth of about 8 h. During the stationary phase the number of viable bacteria declined from 10^9 to $10^{8.5}$ CFU/ml over a period of 10 h.

(iii) Streptococcal preparations. An overnight growth of GBS in Todd-Hewitt broth was adjusted to a concentration of 10^8 CFU/ml by adjusting the optical density at 650 nm to that derived from the standard curve. The bacterial suspensions were centrifuged at $2,000 \times g$ for 15 min, and the supernatant medium was discarded. The bacteria were then washed in MEM by centrifugation at $2,000 \times g$ for 15 min and suspended up to the original volume in MEM. The bacterial suspensions were used immediately or were frozen at −70°C in 1-ml aliquots and stored at that temperature until used. There was no appreciable change in the viability of the organisms as tested by CFU in pour plates after freezing and storage at −70°C for 6 months.

Adherence assay. Bacterial adherence was determined by a modification of the methods of several authors (12, 14, 19, 28). One milliliter of the washed epithelial cells and 1 ml of the washed bacterial suspension in MEM were mixed and incubated at 37°C for 30 min on a tube rotator. The ratio of bacteria to epithelial cells used was 5,000:1. This ratio was based on preliminary experiments which showed that at this ratio the enumeration of adherent streptococci was most reproducible. All experiments were performed in duplicate. The background control (see below) was determined by adding 1 ml of MEM to the epithelial cells instead of bacteria.

After incubation, the mixture was centrifuged twice at $35 \times g$ for 5 min, and the supernatant containing nonadherent organisms was discarded. The recovered epithelial cells were resuspended in 10 ml of fresh MEM, then rinsed with five changes of fresh MEM by filtering through an 8-μm Millipore filter mounted on a syringe, using forward and reverse pressure. The filters were gently inverted and pressed against a precleaned glass slide. The epithelial cells attached to the slide surface were fixed in 95% ethanol for 10 min and Gram stained. The slides were examined at a magnification of ×1,000, and the number of individual cocci, rather than chains, adherent to 50 well-defined epithelial cells was counted. In general the number of cocci per chain varied between 3 and 10.

The background adherence of vaginal flora to vaginal epithelial cells was determined from a suspension of epithelial cells to which no GBS were added. The number of organisms adhering to 50 epithelial cells was counted, and the mean number of adherent bacteria per epithelial cell was calculated. The adherent vaginal flora consisted mainly of low numbers of bacilli.

Materials. Neuraminidase (1 U will liberate 1.0 μmol of *N*-acetyl neuraminic acid per min at pH 5.0 at 37°C, using bovine submaxillary mucin as a substrate), sialic acid (*N*-acetyl neuraminic acid), and bovine submaxillary mucin were purchased from Sigma Chemical Co., St. Louis, Mo.

Statistical analysis. The variation of the distribution was obtained by calculating the standard error of the mean. Significance of differences between means was analyzed using Student's *t* test. The level of significance was considered to be $P < 0.05$.

RESULTS

Reproducibility of adherence test. To test the influence of various parameters on the adherence of GBS to human vaginal epithelial cells, it was essential to determine whether the adherence of streptococcal cells to epithelial cells obtained from the same donor on the same day was reproducible. Samples of five GBS cell suspensions which had been stored at −70°C, each representing a different serotype, were used. Four samples from each suspension were added separately to a tube containing epithelial cells from the same donor and tested for adherence as described above. Table 1 shows the close reproducibility of the adherence test. Although differences in adherence of the various serotypes were encountered, there was no significant difference in the adherence of each of the quadruplicate specimens for each serotype.

Effect of deep freezing of GBS on adherence. Although preliminary studies revealed no change in the viability of GBS following freezing and storage at −70°C, it was important to determine whether freezing affected the adherence of the organisms to epithelial cells. Suspensions of overnight cultures of each of the five GBS serotypes as well as samples of these cultures

TABLE 1. *Reproducibility of adherence of GBS to human vaginal epithelial cells*

GBS serotype	Sample	Adherent bacteria per cell (mean ± SEM)[a]	
		Expt 1[b]	Expt 2[b]
Ia	1	41.0 ± 4.3	15.7 ± 2.0
	2	35.9 ± 2.9	17.5 ± 2.7
	3	39.1 ± 3.6	15.5 ± 1.7
	4	42.7 ± 4.2	16.0 ± 1.8
Ib	1	31.7 ± 3.5	27.3 ± 3.5
	2	39.8 ± 6.0	28.2 ± 3.4
	3	32.4 ± 3.7	27.7 ± 3.1
	4	39.5 ± 5.8	33.9 ± 4.4
Ic	1	42.7 ± 7.9	29.8 ± 3.7
	2	39.8 ± 6.1	28.7 ± 3.4
	3	38.8 ± 5.2	34.3 ± 4.8
	4	38.6 ± 4.8	36.0 ± 4.6
II	1	45.1 ± 4.3	76.4 ± 10.0
	2	43.9 ± 4.2	66.4 ± 7.2
	3	52.2 ± 5.8	71.5 ± 8.6
	4	52.3 ± 5.8	72.8 ± 9.1
III	1	21.2 ± 2.9	11.0 ± 1.4
	2	19.9 ± 2.7	11.3 ± 1.3
	3	27.8 ± 4.2	16.3 ± 3.3
	4	25.7 ± 4.0	17.8 ± 3.7

[a] Derived from counting cocci adherent to 50 epithelial cells per preparation. Background (bacteria adherent to epithelial cells prior to incubation with GBS) for experiment 1 was 3.8 ± 0.68; background for experiment 2 was 3.6 ± 0.90. SEM, Standard error of the mean.

[b] Using duplicate samples of the same streptococcal strains stored at −70°C and epithelial cells from the same donor on different days for each experiment.

frozen at −70°C for 2 and 8 h were tested for adherence to vaginal epithelial cells procured from a donor on the same day. The results of these studies revealed that freezing of streptococci for 2 or 8 h did not alter their capacity to adhere to epithelial cells. This was true for all five strains tested.

Adherence of GBS at various phases of growth. Five strains of GBS representing the five serotypes were cultured at 37°C and harvested after 4, 7, and 10 h (early, mid-, and late log phase) and at 18 and 24 h (early and late stationary phase). The cells were frozen at −70°C immediately upon harvesting. After all the samples were collected, they were tested for their ability to adhere to epithelial cells obtained from the same donor. Adherence of GBS at various phases of growth was almost constant for all five strains. Based on these results, all adherence studies were performed using streptococcal cells from 18-h cultures.

Effect of time and temperature of incubation on adherence. When GBS were incubated at 37°C with vaginal epithelial cells, adherence of the streptococci to the epithelial cells attained maximal levels within the first 15 min of incubation. Testing at further intervals showed no increase in adherence during an additional 3 h of incubation.

GBS serotypes Ia and III adhered to human vaginal epithelial cells even at 4°C. There was significantly higher adherence after 30 min of incubation at 4°C as compared to background (Table 2). Significantly more bacteria adhered at higher incubation temperatures (22 and 37°C) after the same period of incubation.

Effect of pH on adherence. An acidic pH appears to be optimal for the adherence of GBS to human vaginal epithelial cells. Adherence of two serotype III strains in PBS was maximal at pH 5.5 to 6.5 (Fig. 1) and declined significantly at pH values below and above this range. During the course of these studies it was noted that after incubation at a pH below 6, distortion of the epithelial cells could be observed. Adherence of GBS to epithelial cells at pH 7.2 was similar whether HEPES-buffered MEM or phosphate-buffered saline was used to suspend the cells.

Effect of bacterial viability on adherence. The adherence of GBS killed by different methods was compared to that of live organisms. Type III GBS were killed by heating at 65°C for 30 min, by ultraviolet (UV) irradiation, and by incubation with penicillin. UV irradiation was performed by exposing a bacterial suspension (10^8 CFU/ml) in an open petri dish to a UV lamp (Arthur Thomas Co., Philadelphia, Pa.) for 30 min. The distance between the lamp and the surface of the streptococcal suspension was 10 cm, and the radiation at the surface was 1,800 $\mu W/cm^2$. Killing was also achieved by incubating the GBS with 20, 1.25, and 0.75 μg of penicillin

TABLE 2. *Effect of temperature of incubation on the adherence of GBS to human vaginal epithelial cells*

Incubation temp[a] (°C)	Adherent bacterial per cell (mean ± SEM)[b]		Difference
	Ia	III	
Background[c]	4.1 ± 0.76	1.7 ± 0.3	
4	13.4 ± 1.2	15.7 ± 1.2	$P < 0.0001$
22	26.5 ± 4.0	36.5 ± 4.3	$P < 0.0001$
37	48.0 ± 8.4	57.7 ± 8.4	$P < 0.03$

[a] Incubation period at each temperature was 30 min.

[b] Derived from counting cocci adherent to 50 epithelial cells per preparation. SEM, Standard error of the mean.

[c] Bacteria adherent to epithelial cells prior to incubation with GBS.

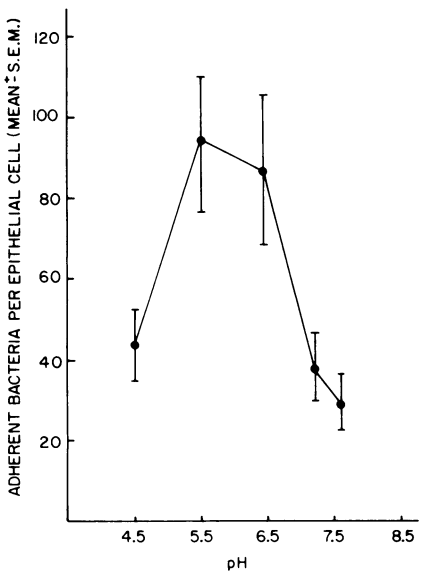

FIG. 1. *Effect of pH on the adherence of GBS to human vaginal epithelial cells. Experiment performed in duplicate. Mean counts were derived from counting cocci adherent to 50 epithelial cells.*

per ml for 18 h at 37°C. The above methods resulted in 90 to 100% reduction in CFU on subculture. Heat treatment of the bacteria significantly decreased the ability of the organisms to adhere to the epithelial cells, whereas UV irradiation and penicillin treatment did not alter adherence to the vaginal epithelium (Table 3).

Epithelial cell viability and adherence. Preliminary studies were performed to determine whether viability of epithelial cells could be assessed by trypan blue exclusion. Cells from several preparations were incubated with trypan blue, and the proportion of cells excluding the dye was determined. Although only 10 to 15% of the epithelial cells from each preparation were capable of excluding trypan blue, GBS consistently adhered to 80 to 90% of the epithelial cells in these preparations. Further studies on the relationship between adherence of GBS and the viability of the cells were performed. The adherence of type III GBS to epithelial cells obtained from the same donor, as well as to suspensions of these cells that were incubated at 65°C for 30 min, was examined. No significant difference was found in the adherence of the cells from the same culture of GBS to the heat-treated and non-heat-treated epithelial cells.

Effect of sialic acid and submaxillary mucin on adherence. Vaginal epithelial cells and type III GBS were incubated in the presence of sialic acid (1 mg/ml) or bovine submaxillary mucin (4 mg/ml) during the performance of the adherence test. In parallel studies, epithelial cells or type III GBS were preincubated separately with sialic acid or mucin, followed by centrifuging the cells prior to performance of the adherence test. Preincubation and adherence were performed in 0.1 M sodium acetate buffer (pH 5.0) for one set of tubes or in MEM with 0.025 M HEPES buffer (pH 7.2) for a duplicate set. Adherence studies performed under the above conditions showed no difference in adherence at either pH between controls and preparations containing sialic acid or mucin.

Effect of treatment of cells with neuraminidase. Vaginal epithelial cells or GBS serotypes Ia, Ib, and III were treated with 0.003 to 0.1 U of neuraminidase (from *Clostridium perfringens*) at 37°C for 60 min in 0.1 M sodium acetate buffer (pH 5.0). The epithelial or bacterial cells were washed and then tested for adherence to untreated cells. Results of a representative experiment are shown in Table 4. As can be seen, there was no alteration in the adherence of GBS to epithelial cells treated with neuraminidase. However, treatment of GBS with neuraminidase resulted in increased adherence of the streptococci to the untreated epithelial cells. Adherence of GBS increased with pretreatment at higher concentrations of enzyme reaching maximum levels at concentrations of neuraminidase of 0.05 U/ml or higher. Similar results were obtained in five separate experiments, which included low buffer control adherence (3 to 9 cocci per epithelial cell) as well as higher buffer control adherence (26 to 33 cocci per epithelial cell). In these experiments pretreatment of GBS with 0.05 to 0.2 U of neuraminidase per ml resulted in approximately a threefold

TABLE 3. *Effect of bacterial killing on adherence of GBS to human vaginal epithelial cells*

Method of killing	Treatment	Adherent bacteria per cell (mean ± SEM)[a]	
		Expt 1[b]	Expt 2[b]
65°C, 30 min[c]	None	50.4 ± 6.4	46.3 ± 7.2
	Heat killed	10.3 ± 1.2	11.6 ± 1.2
UV irradiation,[d] 30 min	None	20.2 ± 2.1	22.1 ± 1.8
	Irradiated	19.7 ± 1.7	20.2 ± 1.8
Penicillin, 18 h[d]	None	24.0 ± 1.8	22.6 ± 1.8
	0.75 μg/ml	20.2 ± 1.8	23.0 ± 1.9
	1.25 μg/ml	24.7 ± 1.7	28.7 ± 2.0
	20.0 μg/ml	27.8 ± 2.0	26.8 ± 1.8

[a] Derived from counting cocci adherent to 50 epithelial cells per preparation. SEM, standard error of the mean.

[b] Using duplicate samples of same streptococcal strains stored at −70°C and epithelial cells from the same donor on the same day for each experiment.

[c] Resulted in 90% reduction in CFU.

[d] Resulted in 100% reduction in CFU.

TABLE 4. *Effect of neuraminidase on adherence of GBS to human vaginal epithelial cells*

Neuraminidase pretreatment (U/ml)	Adherent bacteria per cell (mean ± SEM)[a] after pretreatment of:	
	Epithelial cells	Streptococci
Background[b]	0.08 ± 0.05	0.52 ± 0.2
Buffer control[c]	3.5 ± 0.5	5.5 ± 0.8
0.003	4.7 ± 0.6	6.7 ± 1.2
0.006	3.6 ± 0.5	5.6 ± 1.2
0.012	6.1 ± 0.8	9.5 ± 1.7
0.025	6.0 ± 0.7	11.6 ± 1.5
0.05	5.7 ± 0.6	14.3 ± 2.5
0.1	5.2 ± 0.7	16.8 ± 2.8

[a] Derived from counting cocci adherent to 50 epithelial cells per preparation. SEM, Standard error of the mean.

[b] Bacteria adherent to epithelial cells prior to incubation with GBS.

[c] Acetate (0.1 M; pH 5.0).

increase in adherence over the buffer control.

Treatment of GBS with immune serum. Five serotypes of GBS were treated with an equal volume (1 ml) of undiluted anti-group B immune serum, anti-type immune serum, or normal rabbit serum at 37°C for 30 min using phosphate-buffered saline (pH 7.2). After the cells were pelleted out from the immune or normal rabbit sera, an adherence assay was performed. Coating of streptococci by the antiserum was determined in separate experiments. Using the indirect immunofluorescent antibody technique, cells preincubated with the rabbit group-specific and type-specific antisera reacted positively with fluorescein-conjugated goat anti-rabbit immunoglobulin G. In addition, adherence studies were performed in the presence of immune serum at a final dilution of 1:10. Neither anti-group B nor anti-type antiserum altered the adherence of any of the five serotypes of GBS to human vaginal epithelial cells.

DISCUSSION

The quantitative determination of adherence involves several potential variables prior to the microscopic enumeration of the number of adherent cocci per epithelial cell. It was, therefore, important to attempt to control these variables, starting with the state of growth of the streptococci used in the adherence test. This step required the use not only of organisms that were at a similar physiological state but also organisms consisting of short chains with similar average chain length. One of these requirements was fulfilled when an identical growth curve was obtained for all serotypes of GBS, indicating that different strains grown for the same length of time would be at a similar physiological state. In addition, all strains that were grown for 18 h at 37°C were found to yield chains that were uniformly short, the majority having a length not exceeding 10 cocci. By standardizing these preliminary steps and freezing the cells immediately after harvesting, good reproducibility was achieved in duplicate assays.

Adherence of GBS to vaginal epithelial cells seems to be temperature and pH dependent. Maximal adherence occurred at 37°C, although a significant amount of adherence was observed at 4°C. Adherence peaked at pH 5.5 to 6.5 and declined at pH values below and above this range. The pH of the vaginal secretions during pregnancy is relatively acidic (pH 4 to 6) (8, 9). This implies that the environmental conditions within the vagina in pregnant females favor the adherence of GBS to vaginal epithelial cells.

The data indicating that viability of the streptococcus or the epithelial cell is not a prerequisite for adherence were somewhat unexpected. Killing of GBS by incubation at 65°C for 30 min resulted in a significant decrease in the adherence of the organism to the epithelial cells. However, killing of the streptococci by UV irradiation or with penicillin did not change their adherence capacity. Because of the permeability of epithelial cells in general to vital dyes, it is difficult to assess the viability of these cells with trypan blue unless a clear differentiation can be made between cytoplasmic staining and nuclear staining, which is characteristic of dead cells. However, heating of vaginal epithelial cells at 65°C for 30 min did not affect the adherence of GBS to these cells, supporting the conclusion that both viable and nonviable bacteria can adhere in significant numbers to dead epithelial cells.

The lack of decrease in adherence of bacteria to epithelial cells incubated at 65°C for 30 min suggests that the surface receptor of these cells is heat stable. In contrast, the loss of adherence after heating of GBS at 65°C indicates that a heat-sensitive component of the streptococcal cell or its cell wall is involved in its adherence to epithelial cells. This possibility is supported by the findings that show that UV irradiation, as well as treatment with penicillin, does not reduce the capacity of GBS to adhere to human vaginal epithelial cells. This latter finding contrasts with the data reported by Alkan and Beachey (1) on the inhibition of adherence of group A streptococci treated with penicillin to human oral mucosal cells. In their studies (6, 7, 21, 22) these investigators demonstrated that lipoteichoic acid is responsible for the binding of group A streptococci to epithelial cells. Thus, the loss of adherence of streptococci treated with penicillin

was ascribed to the loss of lipoteichoic acid from the cell wall of the treated streptococcus. Although teichoic acid is also a component of the GBS cell wall, the lack of inhibition of adherence by treatment of streptococci with penicillin concentrations as high as 20 μg/ml suggests that teichoic acid does not play a role in adherence of GBS to vaginal epithelial cells as it does in the adherence of group A streptococci to human oral mucosal cells.

The presence of sialic acid as a major component of the cell wall antigens of all GBS serotypes suggested that an epithelial cell surface receptor for this moiety may be involved in the adherence of GBS to vaginal epithelial cells. However, adherence was not altered by preincubating the cells with sialic acid or by performing the adherence test in the presence of sialic acid. Similarly, mucin, of which sialic acid is also a major component, had no effect on the adherence of GBS to vaginal epithelial cells. Other workers have demonstrated that mucinous glycoproteins of oral secretions inhibit in vitro the adhesion of oral streptococci to oral epithelial cells (30) and to teeth (24). Mucinous glycoproteins are able to competitively inhibit bacterial attachment to the epithelial cells by being structurally similar to the buccal epithelial cell surface components associated with the attachment of oral streptococci. That sialic acid plays no major role in the adherence of GBS to vaginal cells is further supported by the increase in adherence that was observed after digestion of GBS with neuraminidase. This finding suggests that in some of the organisms, the adherence sites on the bacterial cell wall are masked by sialic acid and that these sites become exposed after treatment of the organism with neuraminidase. The latter observation may be of significance in relating the high production of neuraminidase to the enhanced pathogenic potential of GBS as reported by Milligan and his co-workers (20).

Previous work demonstrated that antibody may be able to block the adherence of *Streptococcus sanguis*, *Streptococcus mitis*, and *Streptococcus salivarius* to buccal epithelial cells (29), of *Neisseria gonorrhoeae* to buccal epithelial cells (28), and of *Streptococcus mutans* to glass surfaces (23). Tramont (27) showed that rabbit antisera to piliated *N. gonorrhoeae* inhibited specifically the adherence of these organisms to human buccal epithelial cells. Hamada and Slade (15) showed that the type antigen on the surface of *S. mutans* organisms plays a role in adherence. In the light of these data, the effect of rabbit anti-GBS immune serum on the adherence of these organisms to vaginal epithelial cells was examined in this study. Since Lancefield et al. (18) showed that type-specific GBS antisera afford specific protection to mice challenged with the homologous virulent mouse strains, we tested anti-group as well as anti-type immune sera for their ability to inhibit adherence. No inhibition of adherence was encountered by either anti-group or anti-type antisera. These findings suggest that, in GBS, neither the group-specific nor the type-specific antigenic determinants are involved in the process of adherence of these organisms to vaginal epithelial cells.

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