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# Parameters Affecting the Adherence and Tissue Tropisms of *Streptococcus pyogenes*

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Virulent M protein-containing strains of *Streptococcus pyogenes* were found to adhere well to human pharyngeal cells in vitro. In contrast, an avirulent M- strain and an enteropathogenic *Escherichia coli* strain adhered feebly. When various rat tissues were exposed to mixtures of a virulent *S. pyogenes* strain and an enteropathogenic *E. coli* strain, the relative proportions of the two pathogenic strains recovered from mucosal surfaces differed among the sites studied. *S. pyogenes* cells were found to adhere in higher proportions than enteropathogenic *E. coli* cells to the mucosal surfaces of rat tongues, whereas on surfaces of the urinary bladder, their affinities were reversed. The data indicate that bacterial adherence is influenced by the specificity of both the bacterial and epithelial surfaces, and they suggest that adherence may influence the tissue tropisms of pathogens. Early stationary-phase cells of *S. pyogenes* attached better to epithelial cells than did bacteria in other growth phases. The adherence of *S. pyogenes* cells was impaired by pretreatment with trypsin, wheat germ lipase, Tween 80, Triton X-100, sodium lauryl sulfate, heat at 56 C, anti-group A antiserum, the presence of phospholipids, and preincubation of the epithelial cells with *Streptococcus salivarius* cell walls. Altering the pH or treatment with ethylenediaminetetraacetic acid had no effect on the ability of *S. pyogenes* cells to adhere.

The virulence-related surface antigenic M protein of *Streptococcus pyogenes* has been found to be associated with the organism's ability to attach to mucosal surfaces (3). The extent to which *S. pyogenes* strains are retained on mucosal surfaces influences the degree to which they can colonize. Hence their adherence may be considered a necessary prerequisite for the expression of their virulence. The ability of *S. pyogenes* and other pathogens to adhere to specific tissues should be influenced by factors which include the characteristics of the bacterial and epithelial surfaces and the contents of secretions bathing the particular area. This report describes some of the properties of *S. pyogenes* cells which enable them to attach to epithelial surfaces. Data are also presented which indicate that the relative ability of certain pathogenic species to attach to specific mucosal surfaces varies from site to site in the host, suggesting that these differences are involved in the selective tissue tropisms of infectious bacteria.

## MATERIALS AND METHODS

**Cultures and cultural conditions.** *S. pyogenes* strain C203 (virulent M type 3) was obtained from the American Type Culture Collection. *S. pyogenes* strain C203U (variant of avirulent strain C203S, an M- mutant of C203) was obtained from Isaac Ginsburg, Hebrew University, Jerusalem. *S. pyogenes* strains STA628 (throat strain, M type 12), STA622 (throat strain, M type 1), and PSA54 (skin strain, M type 56) were obtained from Alan L. Bisno of the University of Tennessee, Knoxville. *S. pyogenes* strain SB-1 (throat strain, M type 6) was obtained from Stephan Bellack of the Lincoln State School, Lincoln, Ill. The streptococci were maintained aerobically at 35 C by weekly transfer on tryptic soy agar (BBL) plates containing 5.0% sheep blood. An enteropathogenic *Escherichia coli* strain, Abbotstown (ABB), was obtained from J. B. R. Arbuckle of the Royal Veterinary College, Hertfordshire, England. This strain was maintained aerobically on EMB, MacConkey, and blood agar plates.

**Adherence of S. pyogenes strains and E. coli to human pharyngeal cells in vitro.** The ability of strains of *S. pyogenes* and *E. coli* to attach to human pharyngeal epithelial cells was studied by using an in vitro system described previously (3, 4). Washed bacterial cell suspensions containing $2 \times 10^8$ cells per ml of saline containing 0.01 M phosphate buffer (PBS) were prepared from 20-h Todd-Hewitt broth cultures. These were mixed with washed suspensions containing $10^6$ human pharyngeal epithelial cells per ml which had been collected from one volunteer by gently wiping his posterior pharyngeal wall with

wooden applicator sticks. The mixtures were incubated for 1 h at 35 C in a shaking water bath, after which the epithelial cells were washed free of unattached bacteria. The mean number of bacteria attached per pharyngeal cell was determined by direct light microscope enumeration of 50 epithelial cells. To determine the number of bacteria already attached to the pharyngeal cell surfaces at the time of their collection, samples of the epithelial cell suspension were incubated with buffer instead of mixing them with bacterial suspensions. The effect of tryptic removal of M protein on the adherence of *S. pyogenes* to pharyngeal cells was studied by using methods described previously for human cheek epithelial cells (3).

**Factors influencing the adherence of S. pyogenes to epithelial cells in vitro.** The ability of *S. pyogenes* strains to adhere to buccal mucosa (cheek) epithelial cells under a variety of experimental conditions was studied by using the in vitro methods described above.

**Effect of pH.** Cells of *S. pyogenes* strains C203, STA628, and PSA54 were washed twice and suspended in either acetate or phosphate buffers which ranged in pH from 4.5 to 8.5 before they were incubated with epithelial cells.

**Effect of culture age.** Cultures (500 ml) of strains C203, STA628, and PSA54 were grown at 35 C for 72 h. The optical densities of 2.0-ml samples taken from each culture were determined periodically. Samples (10 ml each) were taken from each culture at 4, 12, 24, and 72 h after inoculation. Bacterial cells from these samples were washed twice in PBS at pH 6.0 and assayed for their ability to adhere.

**Effect of various agents and conditions on adherence.** The effect of various phospholipids on adherence was studied by adding either 80 or 240 $\mu$g of each compound per ml to PBS suspensions of the streptococci prior to mixing them with buccal epithelial cells.

Other factors were studied by pretreating the streptococci for 30 min prior to assessing their ability to adhere to buccal epithelial cells. In addition, the ability of untreated *S. pyogenes* cells to attach to washed epithelial cells preincubated for 30 min with non-trypsin-treated *Streptococcus salivarius* strain 9GS2 cell walls prepared by the method of Salton (13) was studied.

**Relative adherence of S. pyogenes and E. coli to rat mucosal surfaces.** To evaluate the relative ability of *S. pyogenes* and *E. coli* to adhere to oral, intestinal, and urogenital surfaces, methods previously described for studying the adherence of *S. pyogenes* in mice (3) were modified for experimentation with rats. Twenty-h Todd-Hewitt broth cultures of *S. pyogenes* strain SB-1 and *E. coli* strain ABB were harvested by centrifugation, and the cells were washed twice and suspended in PBS at a density of approximately $5.0 \times 10^8$ colony-forming units per ml. A mixed inoculum was prepared by mixing equal volumes of suspensions of the two organisms. Sprague-Dawley germfree rats were inoculated orally and rectally with 1.0 ml of the bacterial mixture by using a polyethylene catheter attached to a syringe. The mixture (1.0 ml) was also injected into the lumen of the nonligated duodenum and into the lumen of the urinary bladder of germfree rats which had been anesthetized with 30 mg of sodium Seconal (Eli Lilly & Co.) per kg of body weight. After 30 min, the rats were killed. Tissue samples were excised and rinsed free of unattached bacteria with PBS. The tongues (oral sample) and distal 2.5 cm of bowel were homogenized for 30 s (Servall Omni-mixer) in 10.0 ml of 0.05% yeast extract solution. Rinsed bladder and duodenal mucosal surfaces were sampled with Calgiswabs (Colab Laboratories, Inc.), which were then dissolved in buffered hexametaphosphate solution. The tissue samples and a sample of the inoculum mixture were serially diluted in yeast extract broth, and appropriate dilutions were plated on 5.0% sheep blood agar plates in duplicate. The plates were incubated aerobically for 2 days at 35 C. The number of colony-forming units of the two species detected in the samples was determined by their distinct colonial morphologies on blood-agar plates. A value for their relative proportions was calculated by multiplying the proportions of each strain detected in the tissue samples by the reciprocal of the proportions of the two strains in the original mixture. This reflects their equal opportunity to adhere to the surfaces studied (16). In addition, a 1.0-ml sample of the inoculum mixture was incubated at 35 C for 30 min with 1.0 ml of either pilocarpine-stimulated rat saliva or human urine.

The relative ability of the two pathogens to adhere to the endocardium of conventional rat hearts was studied. Ten rats were killed, and their hearts were immediately excised and rinsed several times by injection of saline through the openings to the ventricles. The standardized bacterial mixture was introduced in a similar manner. Each heart was submerged in 1.0 ml of saline and incubated at 35 C for 30 min. They were then rinsed free of unattached bacteria, and their endocardial surfaces were sampled with Calgiswabs.

## RESULTS

**Adherence of S. pyogenes strains and E. coli to human pharyngeal cells in vitro.** Cells of M+ virulent *S. pyogenes* strains adhered in significantly higher numbers than cells of *E. coli* strain ABB to human pharyngeal cells in vitro (Fig. 1). Similar to previous studies with human cheek cells (3), virulent M+ *S. pyogenes* throat strains were found to adhere well to pharyngeal epithelial cells in vitro when compared with the feeble adherence of an avirulent M- variant (Fig. 1). Trypsin pretreatment of virulent *S. pyogenes* strains to remove M protein greatly inhibited their ability to adhere to the pharyngeal cells (Table 1). Trypsin treatment of the M- strain C203U had little effect.

**Factors influencing the adherence of S. pyogenes to epithelial surfaces in vitro.** Various substances and treatments were found to alter the ability of *S. pyogenes* strains to adhere to epithelial cells in vitro (Table 2). Compared

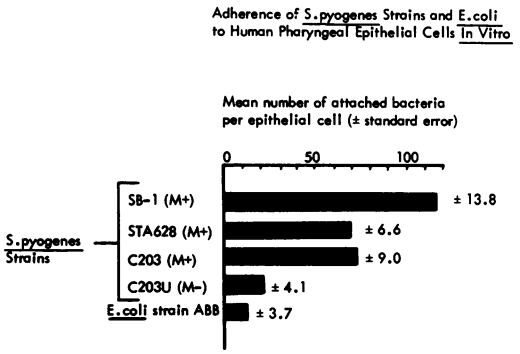


FIG. 1. *Virulent (M+) S. pyogenes strains SB-1, STA628, and C203 attach to human pharyngeal epithelial cells in higher numbers than the avirulent (M−) S. pyogenes strain C203U and the enteropathogenic E. coli strain Abbotstown.*

TABLE 1. *Effect of trypsin pretreatment on the adherence of S. Pyogenes strains to human pharyngeal epithelial cells in vitro*

| *S. pyogenes* strain | Mean no. of attached bacteria per epithelial cell (± standard error) | | Impairment of adherence by trypsin pretreatment (%) |
|---|---|---|---|
| | PBS at pH 8.0[a] (control) | PBS containing 0.5% crystalline trypsin[a] | |
| C203 (M+) | 72.7 ± 9.0 | 11.6 ± 2.1 | 84 |
| STA628 (M+) | 74.8 ± 6.6 | 21.6 ± 1.2 | 71 |
| PSA54 (M+) | 29.1 ± 2.0 | 3.3 ± 0.8 | 89 |
| C203U (M−) | 23.7 ± 4.1 | 21.1 ± 4.9 | 10 |

[a] Pretreatment of *S. pyogenes* cells.

with controls in which 75 to 120 *S. pyogenes* cells generally attached per epithelial cell, the adherence of streptococci pretreated with wheat germ lipase, sodium lauryl sulfate, Tween 80, Triton X-100, anti-group A antiserum, or heat at 56 C was significantly inhibited. Pretreatment of *S. pyogenes* with ethylenediaminetetraacetic acid (EDTA) had no signficant effect on their ability to adhere. Similarly, EDTA treatment of epithelial cells containing attached streptococci for 1 h did not affect the number of adherent *S. pyogenes* cells.

The incorporation of phospholipids into bacteria-epithelial cell mixtures inhibited the adherence of all three *S. pyogenes* strains to some extent (Table 3). The adherence of strain STA628 was the least affected, requiring the higher concentration of each phospholipid to significantly lower the number of attached bacteria. It appears that *S. pyogenes* cells are capable of interacting with several phospholipids commonly found in mammalian cell

TABLE 2. *Effect of various agents and treatments on the relative adherence of S. pyogenes strains to human buccal mucosa epithelial cells in vitro*

| Strain of *S. pyogenes* | Treatment | Relative adherence[a] |
|---|---|---|
| C203 | Heat at 37 C for 30 min (control) | 100 |
| C203 | Heat at 56 C | 26 |
| SB-1 | Heat at 37 C (control) | 100 |
| SB-1 | Heat at 56 C | 25 |
| C203 | PBS at pH 5.0 (control) | 100 |
| C203 | PBS containing 0.2% wheat germ lipase | 24 |
| STA622 | PBS at pH 5.0 (control) | 100 |
| STA622 | PBS containing 0.2% wheat germ lipase | 8 |
| C203 | Buffer (control) | 100 |
| C203 | 1.0% (vol/vol) Tween 80 | 12 |
| C203 | 1.0% (vol/vol) Triton X-100 | 48 |
| C203 | 0.02% Sodium lauryl sulfate | 42 |
| C203 | 0.1 M EDTA at pH 8.0 | 107 |
| C203 | 1.0% Normal rabbit serum (control) | 100 |
| C203 | 1.0% Anti-group A rabbit serum (BBL) | 13 |
| STA628 | 1.0% Normal rabbit serum (control) | 100 |
| STA628 | 1.0% Anti-group A rabbit serum | 36 |

[a] Relative adherence = 100 × (average number of bacteria per cell/average number of control bacteria per cell).

TABLE 3. *Effect of phospholipids on the relative adherence of S. pyogenes strains to human buccal mucosal cells in vitro*

| Bacterial suspension | Amt of compound added (μg/ml) | Relative adherence[a] | | |
|---|---|---|---|---|
| | | C203 | STA628 | PSA54 |
| PBS at pH 6.0 (control) | | 100 | 100 | 100 |
| PBS + phosphatidyl choline | 80 | 5 | 83 | 91 |
| | 240 | 5 | 15 | 0 |
| PBS + phosphatidyl serine | 80 | 12 | 109 | 0 |
| | 240 | 76 | 32 | 0 |
| PBS + phosphatidyl ethanolamine | 80 | 0 | 124 | 22 |
| | 240 | 0 | 54 | 28 |
| PBS + sphingomyelin | 80 | 3 | 62 | 19 |
| | 240 | 0 | 21 | 0 |

[a] Relative adherence = 100 × (average number of bacteria per cell/average number of control bacteria per cell).

membranes, and that differences exist between strains, or M types, in the extent to which these interactions occur. Pretreatment of epithelial cells with cell walls prepared from *S. salivarius*, a species whose adherence is also impaired by phospholipids (5), inhibited the subsequent adherence of control *S. pyogenes* cells by approximately 80%.

When *S. pyogenes* cells were harvested at various phases of growth, it was found that cells entering the stationary phase attached to buccal mucosa cells in highest numbers (Fig. 2). Altering the pH of either acetate or phosphate-buffered cell suspensions of *S. pyogenes* had no significant effect on their ability to adhere.

**Relative adherence of S. pyogenes and E. coli to rat mucosal surfaces.** It was found that mixtures of virulent *S. pyogenes* and *E. coli* cells exhibited dissimilar abilities to adhere to various rat mucosa surfaces in vivo (Table 4). *S. pyogenes* strain SB-1 attached in higher proportions than *E. coli* to the tongue surface of all 10 rats studied; the mean relative proportion of *S. pyogenes* being approximately sixfold greater than that of *E. coli* for this surface. In sharp contrast, their abilities to adhere to the surfaces of the urinary bladder were reversed. On this mucosal surface, the relative proportion of *E. coli* was greater in all 10 rats studied, the mean value for *E. coli* being three times greater than the mean relative proportion of *S. pyogenes*. When samples of the inoculum mixture were incubated with either saliva or urine for 30 min, no significant changes in the proportions of colony-forming units of each organism were detected. These data suggest that the secretions bathing the surfaces studied did not selectively kill one or the other organism. This observation along with the short experimental period (30 min) makes it unlikely that selective growth or death were responsible for the observed population shifts in vivo.

The relative proportions of the two pathogens attached to the mucosa of both the duodenum and lower bowel in vivo as well as to the heart endocardium in vitro were generally similar (Table 4).

## DISCUSSION

The ability of both indigenous and pathogenic bacteria to adhere to a bathed mucosal surface appears to be a prerequisite for their colonization (R. J. Gibbons, New approaches for inducing natural immunity to pyogenic

TABLE 4. *Relative adherence of S. pyogenes SB-1 and E. coli ABB to rat mucosal surfaces*

| Mucosal surface | Mean relative proportions (± standard error) | |
|---|---|---|
| | *S. pyogenes* SB-1 | *E. coli* ABB |
| Tongue (10)[a] | 86.0 ± 11.2 | 14.0 ± 1.5 |
| Urinary bladder (10) | 25.4 ± 4.0 | 74.6 ± 2.9 |
| Duodenum (10) | 40.3 ± 6.6 | 59.7 ± 5.7 |
| Bowel (9) | 45.9 ± 6.1 | 54.1 ± 2.6 |
| Endocardium (10) | 44.3 ± 6.2 | 55.7 ± 3.1 |

[a] Number of rats studied in parentheses.

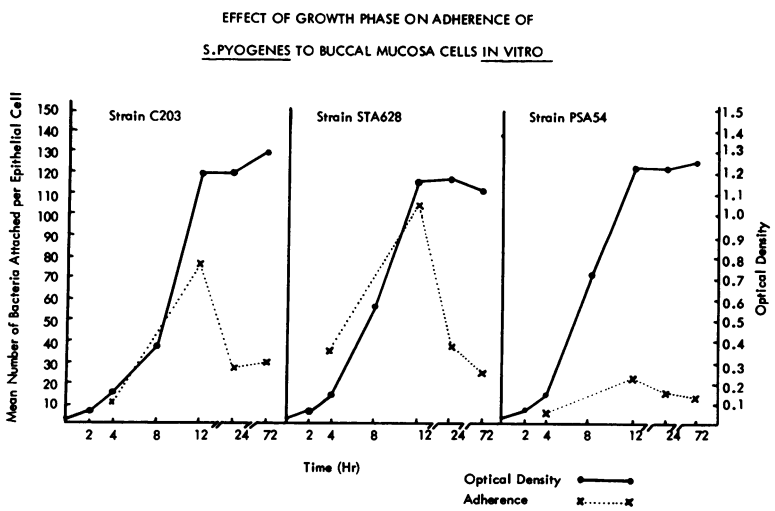


FIG. 2. *Cells of S. pyogenes strains C203, STA628, and PSA54 harvested from early stationary-phase cultures attached in high numbers to buccal mucosa epithelial cells. Skin strain PSA54 adhered feebly when compared with the ability of throat strains C203 and STA628 to attach.*

organisms, in press). A comparison of the abilities of *S. pyogenes* and *E. coli* strains to adhere to human pharyngeal cells demonstrates the specificity involved in these interactions. *S. pyogenes* cells, which naturally colonize the human pharyngeal area, were found to adhere well to human pharyngeal epithelial cells in vitro. In contrast, enteropathogenic *E. coli* cells, which do not commonly colonize the oropharyngeal area, adhered feebly. It is evident from these data that epithelial cells lining the pharynx in man possess specific surface characteristics favoring interactions with components on the surface of *S. pyogenes* cells. On the other hand, the surfaces of virulent *E. coli* cells seem poorly adapted for attaching to human pharyngeal cell receptors.

The contention that attachment selectivity is determined by the specificity of both bacterial and mucosal surfaces was also supported by experimentation with rodents. The present study has demonstrated that pathogenic *S. pyogenes* and *E. coli* strains, which commonly infect dissimilar sites in the human, exhibit distinct differences in their ability to adhere to specific mucosal surfaces of the germfree rat. Similarly, J. B. R. Arbuckle has shown that during experimental infections of piglets, enteropathogenic *E. coli* strains colonize the walls of the small intestinal villi, but not the surface lining the colon (1). Collectively, these findings indicate that the nature of mucosal receptors available for bacterial adherence differ from site to site within the same host. It is apparent that the selectivity of bacterial adherence, through its influence on the colonization of bathed surfaces, must be taken into account to explain the tissue and organ tropisms of pathogenic bacteria. This ecological influence should be similar to that previously described for the intraoral tissue tropisms of indigenous bacteria (4, 8, 9, 16, 17).

Strains of *S. pyogenes* may differ in their ability to infect either skin or pharyngeal surfaces (18). Strains isolated from these sites are often found to be of different serological types, but no explanation has been given for their tissue predelection (18). It is possible that the serological differences, which reflect variations among their surface protein antigens, may influence their affinity for the two sites. In this respect, it is interesting to note that a skin isolate (strain PSA54) adhered feebly to oral mucosal cells relative to throat isolates in the present investigation.

The association of the virulence-related antigen M protein with the adherence of *S. pyogenes* (3) was substantiated by the present study. Virulent strains which contain M protein were found to adhere to human pharyngeal epithelial cells in significantly higher numbers than an avirulent M− strain. Tryptic removal of M protein from virulent strains markedly reduced their ability to adhere to pharyngeal cells.

The adhesion of enteropathogenic *E. coli* strains to the small intestinal wall, their common ecological site for infection, has also been shown to be essential for their virulence (7). Certain virulent *E. coli* strains possess a surface antigen, designated K88, which functions in their adherence to the intestinal mucosa (7) and is absent in avirulent strains. In contrast to the shorter M protein-containing fuzzy structures on the surfaces of *S. pyogenes* cells (15), the K88 antigen is present in long surface filaments on the surface of K88+ *E. coli* cells (14).

Pili have also been associated with the adhesive characteristics of several bacterial species (2). It is likely that the virulence of piliated bacteria is influenced by their ability to adhere to specific mucosal surfaces. For example, pili have been found to be essential for the adherence of virulent gonococci to epithelial surfaces (12). Avirulent strains lack pili and their associated adhesive characteristics (12). Surface components of several bacterial species appear to serve a common ecological function by being essential for adherence. However, their presence in such diverse morphological surface structures suggests that differences in specific adhesive properties of these bacteria may influence the selectivity with which they interact with distinct receptors on dissimilar mucosal surfaces.

Although M protein appears to function in adherence, it is not clear whether it it solely responsible for the actual binding to epithelial surfaces or simply related to the structural integrity of the *S. pyogenes* surface fuzz. However, several findings in this study further clarify characteristics of the bacterial surface, epithelial surface, and secretory environment which may influence the M protein-associated adherence of *S. pyogenes*. Similar to the effect of trypsin, pretreatment with either wheat germ lipase or surface-active detergents impaired *S. pyogenes* adherence. These data suggest that the fuzzy surface of *S. pyogenes* may contain lipids, as reported for the surface coat of *Corynebacterium ovis* cells (6). The adherence of *S. salivarius*, which contains a trypsin-sensitive fuzzy coating morphologically analogous to that of *S. pyogenes* and *C. ovis*, is also inhibited by wheat germ lipase (5). However, such treatment released antigenically active surface components of *S. salivarius* cells (R. J. Gibbons,

unpublished data), a finding not observed for M- protein in this study of *S. pyogenes*.

*S. pyogenes* strains were found to adhere in highest numbers near the end of the logrithmic phase of growth. This may be related to a high degree of M protein production per cell during this period (11). The ability of *S. pyogenes* cells to adhere was inhibited by heat, indicating that the surface components involved in adherence are heat labile. Because the serological reactivity of M antigen is not affected by extraction at high temperatures, these data suggest that adherence is either mediated by a portion of the M protein molecule other than the antigenic determinant or by distinct heat-labile factors closely associated with M protein at the *S. pyogenes* cell surface.

A close similarity was noted among parameters influencing the adherence of *S. pyogenes* cells and those previously reported to effect the attachment of *S. salivarius* to epithelial surfaces (5). Cells of both species attach to oral epithelial surfaces via morphologically analogous, trypsin-sensitive bacterial fuzzy coatings. Their ability to adhere is markedly impaired by treatment with lipase, heat, and phospholipids. These findings, in addition to the fact that both species naturally colonize similar squamous epithelial surfaces lining the oral cavity and pharynx, suggest that *S. pyogenes* and *S. salivarius* cells may interact with similar receptors on epithelial membranes. This contention was further supported by the finding that preincubation of epithelial cells with *S. salivarius* cell walls impaired the subsequent attachment of *S. pyogenes* cells. It is conceivable that competition for receptors by indigenous organisms could account for some of the antagonistic interactions reported to occur between indigenous and pathogenic bacteria. Such a possibility has recently been proposed to account for part of the suppression of *Candida albicans* by the oral flora (10).

Williams and Gibbons have shown that secretory immunoglobulins (Ig) of the IgA type, present in saliva, specifically inhibit the attachment of oral bacteria to epithelial surfaces (19). It was proposed that, in vivo, this mechanism may influence both the serotype conversion of indigenous species and the elimination of pathogens which have induced IgA responses capable of blocking their adherence and facilitating clearance (19; R. J. Gibbons, New approaches for inducing natural immunity to pyogenic organisms, in press). Antibodies specific for M protein have been found to inhibit the adherence of *S. pyogenes* to epithelial cells in vitro (3). However, the nature of the antigens to which secretory antibodies are elicited after natural infections with *S. pyogenes* are not known. It is possible that this response may be predominantly M type-specific, thereby correlating with the well recognized type-specific immunity which develops. In the present study, it was found that group-specific antiserum also inhibits *S. pyogenes* adherence. It may be that antibodies directed against any of several surface antigens are able to hinder the attachment of *S. pyogenes* to epithelial surfaces. If this were to occur in vivo, secretory antibodies directed toward either M protein or less specific surface antigens would be expected to facilitate the clearance of *S. pyogenes* cells and impart immunity.

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