EPITHELIAL CELL BINDING OF
GROUP A STREPTOCOCCI BY LIPOTEICHOIC ACID ON
FIMBRIAE DENUDED OF M PROTEIN*

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Resistance to phagocytosis and epithelial cell binding of Group A streptococci is undoubtedly mediated by surface to surface interactions between host and bacterial cells. Previous ultrastructural studies have demonstrated that the M antigen is associated with surface projections which have been referred to as "fimbriae" (1). The observations were based on ultrastructural studies comparing M-protein-rich and M-protein-negative variants of Group A streptococci, or the regeneration of both fimbriae and M antigen after their complete removal by enzymatic digestion. It could not be determined, therefore, whether or not the fimbriae were composed of M protein alone. Consequently, differences in biological behavior of the streptococcal variants which lack fimbriae cannot be attributed solely to lack of M protein.

It has been suggested that the M protein which is known to be antiphagocytic also mediates binding of Group A streptococci to oral epithelial cells (2). This hypothesis was based on observations employing M-positive and M-negative variants, the limitations of which have been indicated above. In previous studies (3-5), however, we presented evidence to suggest that lipoteichoic acid (LTA)[1] which binds spontaneously to mammalian cell membranes via ester-linked fatty acids may play a central role in the binding of these organisms to mucosal surfaces.

We undertook the present investigation to study the surface ultrastructure in relation to immunochemical and biological activities of Group A streptococci after selective removal of M protein. The behavior of the M-protein-denuded fimbriae of streptococci was compared to that of chemically or enzymatically defimbriated organisms in phagocytosis and epithelial-binding experiments.

Our data indicate that in addition to M protein, LTA can be located on the fimbriae of Group A streptococci and that the role of fimbriae in epithelial cell binding is due to their content of LTA rather than M protein.

Materials and Methods

Culturing and Treatment of Streptococci.   The type 12 and type 24 strains of Group A streptococci used in the present study and the methods of culturing and preserving the organisms were

[1] *Abbreviations used in this paper:* LTA, lipoteichoic acid; MBSA, methylated bovine serum albumin; PBS, 0.02 M phosphate-0.15 M NaCl, pH 7.4; pep 5.8, treated with pepsin at pH 5.8; THB, Todd-Hewitt broth; UV, ultraviolet irradiation.

the same as previously described (6, 7). Streptococci were grown in Todd-Hewitt broth (THB) for 16 h at 37°C and washed twice in ice-cold 0.02 M phosphate-0.15 M NaCl, pH 7.4 (PBS). The organisms were finally resuspended in 10 ml ice-cold PBS and were killed by exposing them to ultraviolet irradiation (UV) for 3 min by the method of Wiley and Wilson (8). Each of the treatments described below employed UV-killed organisms harvested from 40 ml cultures.

Enzymatic and Chemical Treatment of UV-Killed Streptococci.   Organisms were treated with pepsin at pH 5.8 (pep 5.8) as previously described (9), by washing them once in 0.067 M phosphate buffer, pH 5.8, and resuspending in 1 ml of the same buffer containing 20 μg of pepsin (Worthington Biochemical Corp., Freehold, N. J.). The mixture was incubated for 20 min at 37°C at which time the enzymatic activity of pepsin was terminated by adding sufficient 7.5% NaHCO$_3$ to raise the pH to 7.5. Organisms were treated with trypsin by resuspending them in 1 ml, 0.01 M phosphate buffer, pH 8.0, containing 0.1 mg of trypsin (Worthington Biochemical Corp.). Tryptic activity was stopped by adding lima bean trypsin inhibitor (Worthington Biochemical Corp.). Hyaluronidase-treated cells were prepared by incubating organisms in 1 ml PBS (pH 7.4) containing 24 U hyaluronidase. The latter two digestions were carried out at 37°C for 60 min. Streptococci were also treated with hydroxylamine at pH 10.0 as described previously (10, 11) and with hot HCl at pH 2.0 also as described (6, 12). The latter two methods were used in the previous studies to extract M protein from intact streptococci. After each of the enzymatic or chemical treatments, the organisms were chilled in an ice-water bath and were washed twice in ice-cold PBS. The organisms were then ready for use in the procedures described below.

Electron Microscopy.   The variously treated streptococci were fixed for 4 h in ice-cold 5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4, washed twice in the same buffer without glutaraldehyde, postfixed for 1 h in 1.0% osmium tetroxide, and dehydrated in stepwise increasing concentrations (50, 75, 90, and 100%) of ethanol with final dehydration in two 15-min rinses of propylene oxide. The streptococci were then suspended in equal parts of propylene oxide and Maraglas (Ladd Research Industries, Inc., Burlington, Vt.) for 30 min followed by Maraglas alone overnight at 4°C, and were finally embedded in Maraglas in gelatin capsules. In some experiments incubation mixtures of epithelial cells and streptococci (see below) were washed free of nonadherent bacteria and were fixed and embedded. Ultrathin sections (Ultramicrotome; Dupont Instruments, Sorvall Operations, Newtown, Conn.) were placed on naked copper grids, stained for 20 min with 1% uranyl acetate in water and for 5 min in 0.3% lead citrate in 0.1 N NaOH, and examined with an AEI, EM6B electron microscope (AEI Scientific Apparatus Inc., Elmsford, N. Y.) at an electron beam acceleration of 40–60 kV.

Phagocytosis Tests.   Streptococcal phagocytosis tests were performed as has been described in detail elsewhere (13). Briefly, test mixtures contained 0.4 ml fresh, lightly heparinized (10 U/ml) human blood, 0.05 ml of a standardized suspension of streptococci, and 0.05 ml of either normal rabbit serum or M antiserum which was homologous with respect to the serotype of the test organism. After incubation at 8 rpm for 15 min at 37°C, smears were prepared and examined microscopically to estimate percent phagocytosis as previously described (13).

Mucosal Cell-Binding Tests.   Tests of the ability of streptococci to bind to human buccal epithelial cells were performed by the method of Ellen and Gibbons (2) and as previously described (3). Treated and untreated streptococci were resuspended in PBS to a concentration of approximately 2 × 10^8 organisms/ml. Epithelial cells were collected from one of us by scraping the buccal mucosa with wooden applicator sticks. The cells were washed twice in PBS and resuspended in PBS to a concentration of approximately 10^5 cells/ml as estimated by microscopic counts in a hemocytometer. 0.5 ml of the various streptococcal suspensions were mixed with an equal volume of the epithelial cell suspension and incubated for 1 h at 37°C in a rotating apparatus at 20 rpm. After washing the epithelial cells free of unattached streptococci, smears were prepared, stained with crystal violet, and examined by bright-field microscopy. Adhering ability was determined by counting the number of adherent bacteria per 50 epithelial cells. Control cells were incubated with PBS without streptococci to determine the number of bacteria already adhering to the cells before mixing with streptococci. Adherence is expressed as number of bacteria bound per epithelial cell after subtracting the background count. Percent adherence of treated cells is expressed as adherence of treated streptococci divided by the adherence of untreated streptococci, multiplied by 100.

Antisera.   M-protein antisera were raised in rabbits by methods previously described (10, 14).

Capillary precipitin tests of the various streptococcal extracts were performed by the method of Swift et al. (15). Antisera were also raised in rabbits against streptococcal LTA.

LTA was extracted and prepared as previously described in detail (3) and was precipitated with methylated bovine serum albumin (MBSA) (16) at a ratio of one part LTA to one part MBSA (wt/wt). The LTA-MBSA was suspended in PBS to a concentration equivalent to 0.25 mg/ml of LTA and was emulsified with an equal volume of Freund's incomplete adjuvant and then mixed with an equal volume of 2% Tween 80 (Dr. Robert Jackson, personal communication). Rabbits were injected subcutaneously with the emulsion corresponding to a dose of 100 μg LTA in a total vol of 1 ml. Anti-LTA antisera were tested by the passive hemagglutination of human group O erythrocytes coated with LTA as previously described (3).

Results

In a previous study (9) we reported a method of extracting M protein by treating intact M-rich streptococci with dilute solutions of pepsin (20 μg/ml) at pH 5.8. In the present study we examined the ultrastructure as well as the biological reactions of the streptococci that had their M protein removed by such mild pepsin digestion in comparison to streptococci treated by other enzymatic and chemical agents. In order to assure that streptococci would not regenerate new surface fimbriae that possessed M protein, the organisms were killed by mercury-arc irradiation (UV killed) before treating them with the various agents. Such brief UV had no effect on the ability of streptococci to bind to oral epithelial cells or to resist phagocytosis. Wiley and Wilson (8) had previously demonstrated that Group A streptococci killed in this way by UV retained their resistance to ingestion by phagocytes.

Ultrastructure of Treated Streptococci. The streptococci treated with pepsin (pH 5.8), hydroxylamine, or hyaluronidase retained their fimbriae (Fig. 1 *b–d*), whereas the organisms treated with hot HCl or trypsin lost virtually all of their fimbriae (Fig. 1 *e* and *f*). The hyaluronate capsule although not ultrastructurally visible could be demonstrated by India-ink preparations to be present only in untreated and pep 5.8 streptococci.

M-Protein Content of Treated Streptococci. Hot (95°C) HCl at pH 2.0 is a well-known method for extracting M protein from Group A streptococci. We, therefore, used this method to examine for the presence or absence of M protein in streptococci after the various treatments. The HCl extract of pep 5.8 streptococci lacked any detectable M protein; extracts that had been concentrated 10-fold showed only trace amounts of M precipitin activity, but no opsonic inhibitory activity (13), indicating that virtually all M protein had been removed from the surface fimbriae (Table I). In contrast, M protein was present in HCl extracts of fimbriated streptococci pretreated with hydroxylamine or hyaluronidase. HCl extracts of the nonfimbriated streptococci pretreated with trypsin were devoid of any detectable M protein (Table I).

Biological Behavior of Treated Streptococci. Although the importance of fimbriae-associated M protein in mediating resistance to phagocytosis is well recognized, its possible role in binding of streptococci to epithelial cells has not been adequately answered. There is evidence (2), however, to suggest that surface fimbriae of streptococci are intimately involved in the binding process as is illustrated by electron microscopy of streptococcal attachment to mucosal cells (Fig. 2). Since pepsin digestion at pH 5.8 was the only treatment employed in the
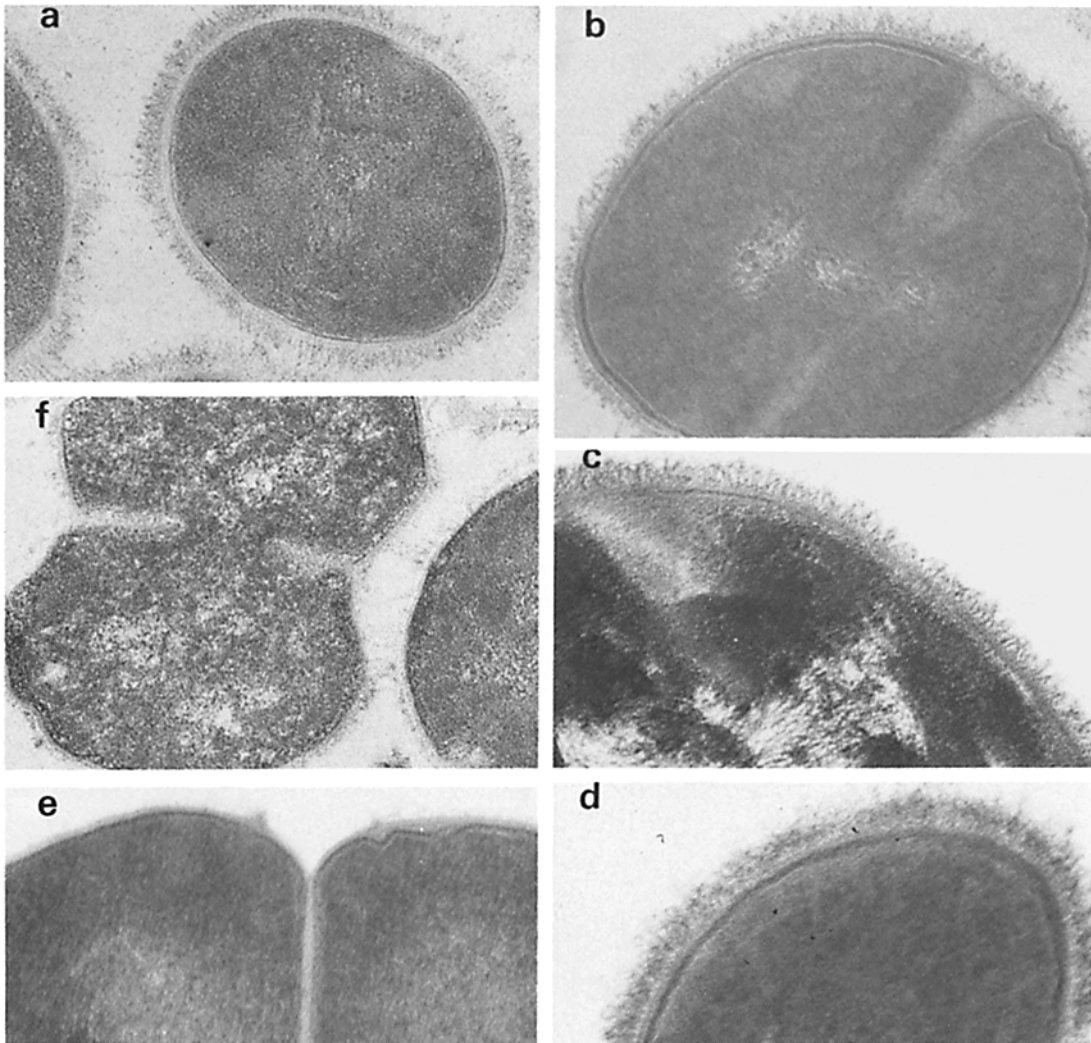
Fig. 1. Cross-sections of UV-killed Group A streptococci demonstrating the presence of surface fimbriae in untreated control cells (a), and the preservation of the fimbriae after removal of M protein by peptic digestion at pH 5.8 (b). Fimbriae were also retained after treatment with hydroxylamine at pH 10.0 (c), or digestion with hyaluronidase to remove hyaluronic acid capsule (d). In contrast, digestion with trypsin (e), or extraction with hot HCl (f) removed virtually all of these surface projections.

above experiments that dissociated the M antigen from the intact fimbriae, we used the pep 5.8 streptococci to assess the epithelial-binding activity of fimbriated organisms devoid of M protein in comparison with (a) untreated, fimbriated organisms that contain intact M protein and (b) trypsin or hot HCl-treated organisms which lacked both M protein and fimbriae. The latter defimbriated organisms lost both resistance to phagocytosis and ability to bind to oral mucosal cells (Tables II and III). In contrast, the organisms treated with pepsin at pH 5.8, which retained their fimbriae also retained their mucosal-binding

TABLE I

Effect of Treating UV-killed Streptococci with Various Agents on Extractability of M Antigen with HCl

Streptococci extracted with HCl* after treatment with:	Presence of fimbriae before HCl extraction	Precipitin reactions of HCl extracts:	
		Crude	10× ‡
Pepsin, pH 5.8	+ §	− ‖	±
Trypsin, pH 8.0	−	−	−
Hyaluronidase, pH 7.4	+	+ + + +	ND¶
HCl, pH 2.0	±	±	+ +
Hydroxylamine, pH 10.0	+	±	+ +
Control buffers**	+	+ + + +	ND
Untreated	+	+ + + +	ND

* Streptococci were extracted with HCl, pH 2.0, at 95°C for 10 min. (See text.)

‡ 10×, 10-fold concentrate of crude extract prepared by ultrafiltration.

§ State of surface fimbriae: +, visibly intact; ±, sparse; −, absent.

‖ Precipitin reactions: −, no visible precipitate after 24 h; ±, visible precipitate but less than 1 mm in capillary tube; and +, 1 mm of precipitate up to + + + +, 4 mm or greater.

¶ ND, not done.

** Control buffers consisted of each of the buffers without the respective enzyme or hydrolytic agent. The results are combined since there was no detectable difference among the various buffers.

ability (Table III) although they lost their M protein and resistance to phagocytosis (Table II). Moreover, type-specific M antibody which enhanced phagocytosis of untreated and hydroxylamine- or hyaluronidase-treated organisms (which retained extractable M protein) did not further enhance phagocytosis of the pep 5.8 organisms (Table II), indicating that these fimbriated streptococci which lack M protein cannot be opsonized by homologous M antibody.

These results suggest that binding of Group A streptococci to oral epithelial cells is not mediated by M protein. In the following studies we present evidence that a portion of the streptococcal LTA resides on the cell surface whether or not the fimbriae are denuded of M protein and that the surface LTA binds the streptococci to the mucosal cells.

Inhibition of Epithelial Cell Binding of Streptococci by Anti-LTA. Each of the homologous or heterologous M-type-specific or Group A-specific antisera inhibited epithelial binding of type 12 Group A streptococci (Table IV). Upon further examination, however, we found that each of the inhibitory antisera contained anti-LTA titers of 1:128 or greater. Absorption of the antisera with LTA-coated erythrocytes (see Materials and Methods) abolished the binding-inhibitory effect of each of the sera. Absorption of the antisera with LTA had no effect on the respective M-type-specific or Group A-specific precipitin reactions (Table IV).

An antiserum prepared against purified LTA inhibited binding of streptococci even though it lacked any detectable group-specific or type-specific antibodies (Table IV). Moreover, the anti-LTA inhibited adherence of pep 5.8 streptococci to a similar degree (69%, data not shown in table), indicating that sufficient
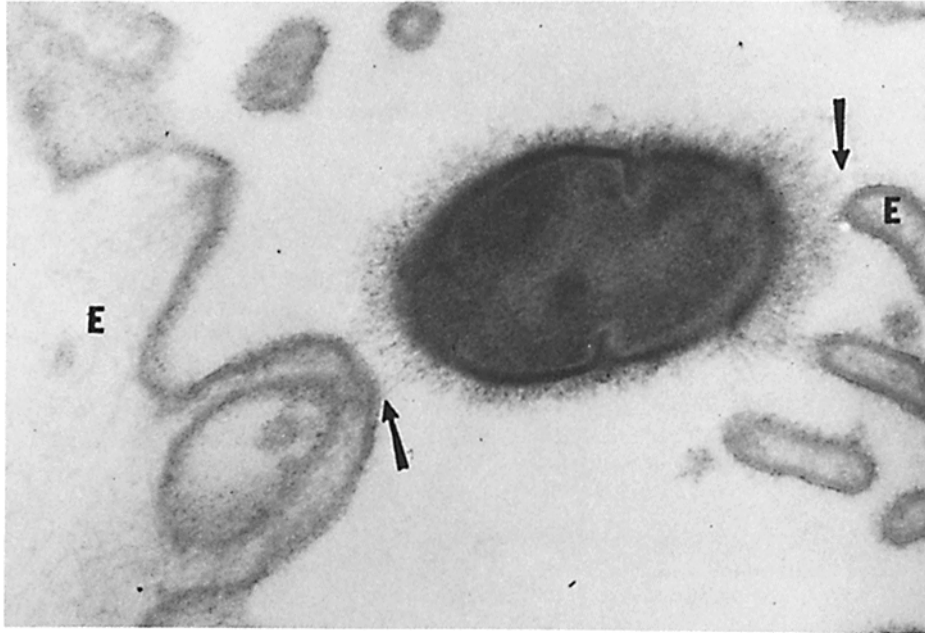
FIG. 2. Fimbriae radiating from Group A streptococcus to membrane (arrows) of human oral epithelial cell (E).

TABLE II

Effects of Treatment with Various Agents on the Resistance of UV-Killed Streptococci to Phagocytosis

Treatment of UV-killed streptococci	% Phagocytosis* in presence of:	
	NRS‡	Anti-M24‡
Untreated	0	76
Pepsin, pH 5.8	69	65
Pepsin, pH 5.8, + hyaluronidase	87	93
Trypsin	100	100
Hyaluronidase	19	58
Hydroxylamine, pH 10.0	40	93
HCl, pH 2.0	87	88

* Expressed as number of neutrophiles per 100 counted cells containing one or more cocci after incubation for 30 min.

‡ NRS, normal rabbit serum; and anti-M24, rabbit antiserum prepared by immunizing rabbits with whole streptococci as previously described.

LTA is retained on the surface of these organisms to mediate binding to epithelial cells. The inhibitory effects of the LTA antiserum were abolished by absorption with purified LTA (Table IV).

Immunofluorescence of Oral Epithelial Cells Treated with LTA. Oral epithelial cells were incubated with 1 mg/ml of either (*a*) purified LTA or (*b*)

TABLE III
*Effects of Treatment with Various Agents on Adherence of UV-
Killed Streptococci to Epithelial Cells*

Treatment of streptococci	% Adherence*
Untreated	100
Pepsin, pH 5.8	92
Trypsin	0
Hyaluronidase	100
HCl, pH 2.0	0
Hydroxylamine, pH 10	100

* See Materials and Methods for derivation.

TABLE IV
*Removal of the Inhibition Effects on Binding from Various Group A Streptococcal
Antisera by Absorption with LTA*

Antisera	Reactions of unabsorbed antisera			Reactions of antisera absorbed with LTA		
	% Inhibition of adherence*	anti-LTA titer‡	precipitin reactions§	% Inhibition of adherence*	anti-LTA titer‡	precipitin reactions§
Anti-Group A	37	1:256	+ +	0	<1:2	+ +
Anti-M12	43	1:128	+ + + +	0	<1:2	+ + + +
Anti-M24	53	1:256	+ + + +	0	<1:2	+ + + +
Anti-LTA	73	1:1,280	–	0	<1:2	–
NRS	0	1:8	–	ND	ND	ND

* % inhibition of binding, 100 – % adherence.
‡ Results recorded as highest twofold dilution of antiserum that agglutinated erythrocytes coated with LTA.
§ Capillary precipitin reactions with standard hot acid extracts prepared from the homologous strains of streptococci.

purified type 24 M protein (10) that lacked LTA. The cells were then treated with anti-LTA or LTA-absorbed anti-M24 sera, respectively. Both cell preparations were then treated with fluorescein-conjugated goat antirabbit globulin and examined by ultraviolet microscopy. The epithelial cells treated with LTA (Fig. 3 *a*), but not with M protein (Fig. 3 *b*), were strongly fluorescent, indicating the spontaneous affinity of LTA, but not M protein for cell membranes.

Extraction of Surface LTA from Untreated and Pep 5.8 Streptococci.   LTA was extracted from the intact surface of untreated or pep 5.8 streptococci with acetate buffer at pH 6.0 as described by McCarty (17). The anti-LTA reactivity and spontaneous binding to erythrocytes of the LTA extracted from pep 5.8 organisms was similar to that extracted from untreated organisms, indicating that LTA was retained on the surface of the organisms after they had been denuded of M protein.

Further evidence that cell wall substances other than LTA are not involved in the adherence of streptococci to epithelial cells was obtained in an experiment in which oral epithelial cells were preincubated with various cell wall components of Group A streptococci. Binding of streptococci to the treated cells was inhibited
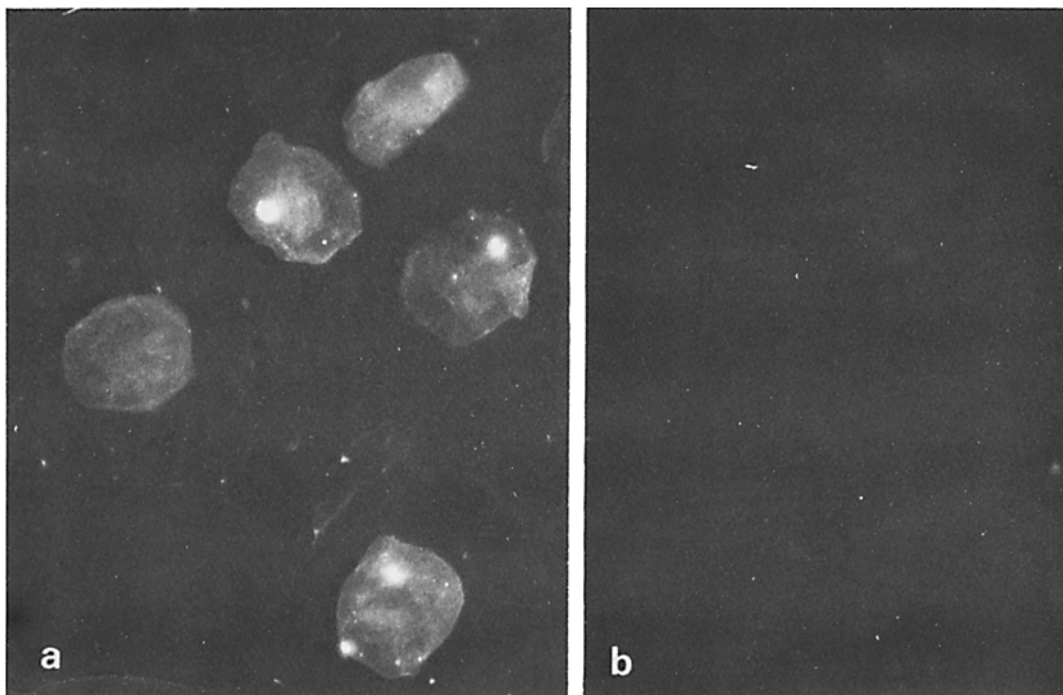
Fig. 3. Human buccal epithelial cells treated with 1 mg/ml LTA)), or 1 mg/ml purified type 24 M protein (b). The epithelial cells were then treated with a 1:5 dilution of rabbit antiserum to the respective antigen followed by fluorescein-conjugated goat antirabbit globulin.

by LTA, but not by M protein, C carbohydrate, peptidoglycan, or by lipopolysaccharide of *Serratia marcescens* or *Escherichia coli* (Table V).

Immunoelectron Microscopy of Surface LTA. Type 12 streptococci were grown for 16 h in THB, washed, and incubated with antiserum against purified LTA followed by goat antirabbit IgG conjugated with ferritin. Examination of whole streptococci demonstrated diffuse labeling of the surface with anti-LTA (Fig. 4 a). The surface labeling was abolished by absorbing the anti-LTA with erythrocytes coated with purified LTA (Fig. 4 b). These results further confirm that a portion of streptococcal LTA is located on the surface of Group A organisms.

Discussion

One of the most intriguing findings in the present study is that M protein can be dissociated from intact fimbriae of Group A streptococci. It has been long recognized that surface M protein is a major determinant of virulence with respect to its role in the pathogenicity of streptococci for mice and in resistance of the organisms to ingestion by phagocytes (18-20). Moreover, the role of M protein in the initial step of the infectious process, namely adherence of the bacteria to mucosal surfaces has been recently suggested (2).

Electron microscope studies by Ellen and Gibbons (2) and in the present study, suggest that fimbriae are intimately involved in the binding of strepto-

TABLE V
Effect of Preincubating Human Buccal Epithelial Cells with Cell Wall Substances upon Epithelial Cell Binding of Group A Streptococci

Mucosal cells pretreated* with:	% Inhibition of epithelial cell binding of streptococci
Lipoteichoic acid	78
M protein	0
C carbohydrate	0
Peptidoglycan sonicate	0
Lipopolysaccharide‡ (*S. marcescens* and *E. coli*)	0

* Epithelial cells were preincubated for 30 min at 37°C with each substance at a final concentration of 1 mg/mg in PBS.

‡ Lipopolysaccharides from these organisms obtained commercially (Difco Laboratories, Detroit, Mich.).

cocci to mucosal cell membranes. Previous studies (1) have indicated that the M antigen is associated with such fimbriae. The mere presence of M protein however, does not necessarily indicate that this substance is ultimately required as has been implied for the binding of the bacteria to epithelial cells, although it has been reported (2) that M-protein-producing streptococci adhere to epithelial cells in greater numbers than a non-M-protein-producing strain. Ultrastructural studies of the nonadhering, M-negative strain to determine the presence or absence of fimbriae were not reported; it has been shown in other studies (1, 21) that such variant streptococci lack fimbriae.

The ability in the present study to dissociate M protein from fimbriae has made it possible to dissociate determinants of fimbriae involved in resistance to phagocytosis from those involved in epithelial binding. The data in this report together with our previously reported data (3, 4) support the hypothesis that the antiphagocytic M substance is not involved in binding streptococci to human mucosal cells because (a) M protein or M-protein antibody was not able to inhibit adherence and (b) fimbriated streptococci that were denuded of M protein retained their adhering ability. It is of interest that dissociation between resistance to phagocytosis and epithelial cell binding has recently been reported for pilated gonococci (22) and pilated *Proteus mirabilis* (23), suggesting that the distinction between the components involved in these two virulence mechanisms may occur as a more general phenomenon among various bacterial pathogens.

The distinction between the components involved in the two virulence mechanisms is consistent with the in vivo studies of Krause and Rammelkamp (24) who were able to colonize the oral mucosal surfaces of monkeys with a non-M-producing variant of type 14 streptococci. Furthermore, epidemiological studies (25, 26) have consistently demonstrated that over one-half of the streptococcal strains isolated from various human populations lacked detectable M antigens. The possibility that some of these isolates represented new M serotypes was excluded by their inability to resist phagocytosis (25). Whether or not the
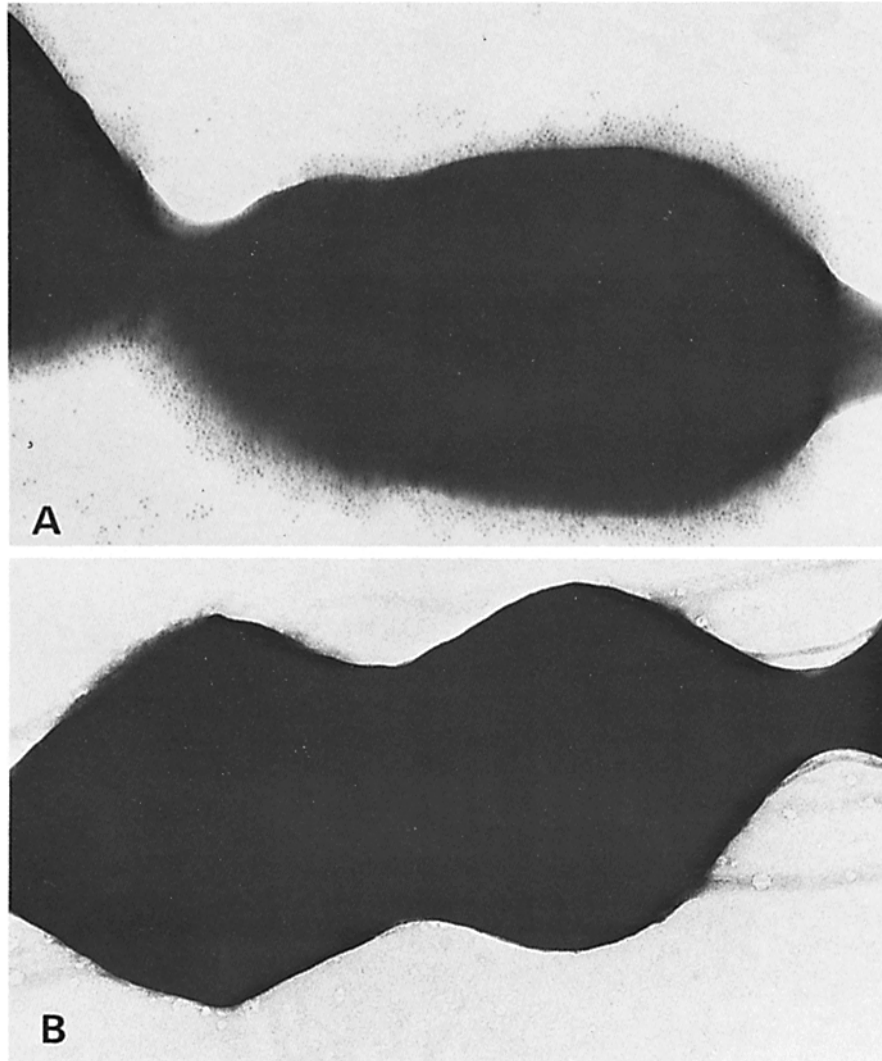
Fig. 4.   Electron micrographs of whole Group A streptococci treated with unabsorbed anti-LTA (A), or with anti-LTA absorbed with erythrocytes coated with purified LTA (B), followed by treatment with ferritin-conjugated goat antirabbit globulin.

isolates in these epidemiological studies possessed surface fimbriae even though they lacked M protein was not investigated.

Our study suggests that a fimbriae-associated substance other than M protein mediates mucosal cell adherence of streptococci; streptococci that were chemically or enzymatically defimbriated lost their ability to bind to mucosal cells. We have previously presented evidence to suggest that Group A streptococci bind to epithelial cells via fatty acids which are ester-linked to glycerol teichoic acid. Our data in the present study further support the hypothesis that lipoteichoic acid is centrally involved in the epithelial cell binding of streptococci, and is associated with surface structures of Group A organisms. Together with our previous studies (3–5) we have demonstrated that (a) binding of streptococci to

oral mucosal cells is blocked by preincubating epithelial cells with LTA or by preincubating streptococci with LTA antibody present in the sera of immunized rabbits and man, (b) in agreement with the original observations of McCarty (17) LTA can be extracted from the surface of intact Group A streptococci, and (c) LTA can be located on the surface of Group A streptococci by immunoelectron microscopy.

The surface location of a portion of Group A streptococcal LTA is consistent with the recent findings of Wicken and Knox (27) and Birdsell et al. (28) who similarly demonstrated that LTA could function as a surface component in other gram-positive bacteria; these included *Lactobacillus casei, L. fermenti,* and *Bacillus subtilus.*

In our preliminary screening studies (5) M-negative variants of Group A streptococci adhered to mucosal cells equally as well as the M-positive strains. Ellen and Gibbons (2), however, demonstrated that a single M-negative variant adhered less well than M-positive strains. Whether or not streptococcal strains which have lost surface LTA and epithelial cell-binding ability may emerge during laboratory culturing or naturally, similar to the well-documented emergence of strains which lack M protein and resistance to phagocytosis (18, 20), requires further investigation. In any case, the ability to distinguish between bacterial surface components involved in mucosal binding and those involved in other virulence mechanisms, such as resistance to phagocytosis, should provide methods for investigations of the significance of colonization by Group A streptococci in face of host immunity.

Summary

Group A streptococci were treated with various enzymatic and chemical agents in an attempt to dissociate the type-specific M protein from intact surface "fimbriae." Mild peptic digestion at pH 5.8, which was previously shown to extract serologically active M antigen from intact streptococci had little visible effect on the fimbriae even though virtually all of the M protein was removed as demonstrated by (a) increased susceptibility to phagocytosis, (b) lack of opsonic effect of homologous M antibody on the treated streptococci, and (c) loss of HCl-extractable M protein. These fimbriated streptococci which lacked M protein adhered to human oral mucosal cells equally as well as untreated, fimbriated organisms which retained their M protein. Removal of both fimbriae and M protein by digesting organisms with HCl at pH 2.0 at 94°C or with trypsin abolished their ability to bind mucosal cells. Electron microscopy of streptococci bound to epithelial cells demonstrated fimbriae radiating from the surface of the organisms to the membrane of the epithelial cells.

It is apparent, therefore, that the determinants of streptococcal fimbriae involved in resistance to phagocytosis can be dissociated from those involved in epithelial cell binding. These results are consistent with our previous studies which suggested that fatty acids ester linked with glycerol teichoic acid rather than M protein of streptococci binds the organisms to epithelial cells.

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