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Research Article

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Hyaluronic Acid Capsule Modulates M Protein–mediated Adherence and Acts as a Ligand for Attachment of Group A *Streptococcus* to CD44 on Human Keratinocytes

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Abstract

We used wild-type and isogenic mutant strains of group A *Streptococcus* (GAS) that expressed M protein, capsule, or both to study the function of M protein and the hyaluronic acid capsular polysaccharide in attachment of GAS to human keratinocytes. Types 6 and 24, but not type 18, M protein were found to mediate attachment of GAS to soft palate or skin keratinocytes, but this interaction was prevented by the hyaluronic acid capsule on highly encapsulated, or mucoid, strains. Monoclonal antibody to CD44, the principal hyaluronic acid–binding receptor on keratinocytes, inhibited attachment of both highly encapsulated and poorly encapsulated wild type strains of GAS, but not the attachment of acapsular mutants. Transfection of K562 cells with cDNA encoding human CD44 conferred the capacity to bind each of six wild-type strains of GAS, but not to bind acapsular mutants. Because, in contrast to other potential adhesins, the group A streptococcal capsule is both highly conserved and surface-exposed, it may serve as a universal adhesin for attachment of diverse strains of GAS to keratinocytes of the pharyngeal mucosa and the skin. (*J. Clin. Invest.* 1998. 101:1708–1716.) Key words: keratinocyte • group A *Streptococcus* • hyaluronic acid • CD44 • adherence

Introduction

Group A *Streptococcus* (GAS)[1] causes pharyngitis and infections of the skin and soft tissues. These common syndromes are occasionally complicated by severe invasive infection, by the streptococcal toxic shock syndrome, or by the postinfectious sequelae of acute rheumatic fever or glomerulonephritis. GAS infection begins with the association of the bacteria with the pharynx or external skin. Attachment of GAS to the epithelial surface at these sites is thought to represent a critical first step in establishing infection.

Investigations of GAS adhesion suggest that several different GAS surface molecules may participate in attachment of the bacteria to various eukaryotic cells. Hasty et al. (1) have suggested a two-step model of GAS attachment whereby a low affinity primary adhesin, lipoteichoic acid, mediates a weak initial attachment followed by a stronger and more specific one, such as by a fibronectin-binding protein or the GAS M protein. In their studies, addition of purified lipoteichoic acid blocked attachment of GAS to buccal cells and to a squamous carcinoma cell line, HEp-2. Some strains of GAS express one of at least three different fibronectin-binding proteins that have been shown to participate in attachment of certain GAS strains to respiratory epithelial cells (2), Langerhans' cells (3), or HEp-2 cells (4, 5). These proteins, however, are absent on some clinically important serotypes of GAS, including M types 1 and 3, two of the most common serotypes among pharyngitis and invasive infection cases in the United States (6).

M protein, the major GAS surface protein implicated in resistance to phagocytosis, has been reported to mediate attachment of GAS to human epidermal keratinocytes. In studies of type 6 GAS and isogenic mutants derived from it, membrane cofactor protein (CD46) was identified as a receptor for M protein on HaCat cells, a human keratinocyte line, and in human skin sections (7). However, in earlier studies, M protein did not appear to be involved in adhesion to human tonsillar epithelial cells derived from organ cultures (8). In studies of an M protein–deficient mutant derived from a type 24 strain of GAS, Courtney et al. (9) found that M protein participated in attachment of GAS to HEp-2 cells, but not to human buccal cells in vitro. The results of these studies suggest that the role of M protein and other surface molecules as adhesins for GAS is complex and may depend upon serotype- or strain-specific differences in the proteins themselves, exposure or masking of potential adhesins by other GAS surface molecules, and the nature of the target epithelial cells.

The hyaluronic acid capsule of GAS has been shown to protect the organisms from ingestion both by phagocytes and epithelial cells, and to enhance virulence in mouse models of lethal systemic infection, upper airway colonization, and pneumonia (10–14). However, Bartelt and Duncan (15) found that hyaluronidase treatment of certain strains of GAS increased adherence to a human pharyngeal carcinoma cell line, and suggested that the capsule impaired attachment. The goal of these investigations was to reexamine GAS attachment to human epithelial cells, focusing on the role of the hyaluronic acid capsule in bacterial adherence to keratinocytes, the major cell type of human pharyngeal epithelium and external skin. To understand the interactions between the capsule and M pro-

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1. *Abbreviation used in this paper:* GAS, group A *Streptococcus.*

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tein in adherence, we studied isogenic GAS strains deficient in capsule, M protein, or both in the backgrounds of three different GAS serotypes. The results of these studies indicate that the role of M protein as an adhesin depends on M type, on masking effects of the capsule, and on the tissue source of the target epithelial cells. A major new finding of this investigation is that, in addition to modulating binding interactions mediated by other bacterial surface molecules, the hyaluronic acid capsule itself acts as an adhesin for attachment of diverse strains of GAS to the hyaluronic acid-binding glycoprotein CD44 on human keratinocytes.

Methods

Bacterial strains and cultures. The GAS strains used in this study, their relevant features, and the references or sources from whom they were obtained are listed in Table I. GAS were grown in Todd-Hewitt broth supplemented with 0.5% yeast extract or on trypticase soy agar containing 5% defibrinated sheep blood. For bacterial attachment studies, GAS were grown in broth to midexponential phase (A_{650nm} = 0.12–0.14). *Escherichia coli* DH5α was grown in Luria Bertani broth or on Luria Bertani agar. When required, antibiotics were added to growth media: erythromycin at 1 μg/ml for GAS or 150 μg/ml for *E. coli*, and kanamycin at 500 μg/ml for GAS or 50 μg/ml for *E. coli*.

Construction of GAS strain 24-4Ω3, an acapsular derivative of strain M24-Ω3. The transposon Tn*916* insertion mutation in the *hasA* gene (encoding hyaluronate synthase) was transferred by transduction from acapsular mutant strain TX72 to GAS strain M24-Ω3 using bacteriophage A25, as described previously (16), to create strain 24-4Ω3, an M−, capsule− derivative of strain Vaughn.

Construction of 003ΔhasA. A 657-basepair internal fragment of the *hasA* gene was amplified by PCR from GAS chromosomal DNA using oligonucleotide primers, each of which included a 5′ extension containing either a BamHI or PstI restriction endonuclease site. The PCR product was cloned initially in plasmid pWKS30 (16a), then the insert was cut out of the recombinant plasmid with BamHI and Asp718 and cloned in the temperature-sensitive shuttle vector pJRS233 (17). The ΩKm-2 element encoding kanamycin resistance (18) was inserted into an internal BglII site of the cloned *hasA* gene fragment to form the plasmid phasAΩ. Plasmid phasAΩ was introduced into GAS strain DLS003 by electroporation as described by Caparon and Scott (19). To integrate the plasmid into the GAS chromosome, thereby disrupting expression of *hasA*, a transformant was grown in broth culture at 30°C for 16 h in the presence of erythromycin, then an aliquot of the culture was diluted in fresh medium and incubated overnight at 37°C to prevent replication of the nonintegrated plasmid. This step was repeated once, then aliquots were spread on erythromycin-containing agar and integrants were selected as colonies that grew at 37°C. Integration of the plasmid within the chromosomal *hasA* locus was confirmed by PCR using a primer from the 3′ portion of the ΩKm-2 element (5′-CGGTTTACAAGCATAAAGCTTGC-3′) and a primer from the 3′ terminus of the *hasC* gene, downstream of *hasA* (5′-CCCCCCAAGCTTCCAACATCGTAACGATTGCC-3′). Amplification of the acapsular mutant strain 003ΔhasA chromosomal DNA yielded a product of the expected size; control reactions using DLS003 chromosomal DNA or plasmid phasΩ DNA as template were negative.

Table I. GAS Strains Used in this Study

Strain	Relevant features	Reference or source
M type 6 GAS and isogenic mutant		
JRS4	M6 wild type strain	(37)
JRS145	M− derivative of JRS4	(38)
M type 24 GAS and isogenic mutants		
Vaughn	M24 wild-type strain	(9)
24-4	Capsule− derivative of Vaughn	(16)
M24-Ω3	M− derivative of Vaughn	(9)
24-4Ω3	M−, capsule− derivative of Vaughn	This study
M type 18 GAS and isogenic mutants		
87-282	M18 wild-type strain	(10)
TX72	Capsule− derivative of 87-282	(16)
282KZ	M− derivative of 87-282	(39)
TX74	M−, capsule− derivative of 87-282	(39)
M type 3 GAS and isogenic mutant		
DLS003	M3 wild-type strain	Dennis Stevens
003ΔhasA	Capsule− derivative of DLS003	This study
Additional wild-type GAS strains		
950358	M1 wild-type strain	Edward Kaplan
950802	M1 wild-type strain	Edward Kaplan
94467	M3 wild-type strain	Edward Kaplan
950771	M3 wild-type strain	Edward Kaplan
SS-644	M27 wild-type strain	Richard Facklam

Antibodies. mAbs 4A4 and 8d8 to human CD44 have been described previously (20, 21). Both mAbs bind to CD44 on keratinocytes, but do not block hyaluronic acid binding; mAb 4A4 does inhibit CD44-mediated attachment of lymphoid cells to immobilized hyaluronic acid (20). mAb IM7.8.1, provided by Robert Hyman (Salk Institute, San Diego, CA), is a rat/mouse hybridoma to CD44 that blocks hyaluronic acid binding to CD44 on certain cell types, including keratinocytes (22–24; our unpublished observations). Rabbit antiserum to the GAS group A carbohydrate was a gift of Joseph Crabb (Immucell Corp, Portland, Maine) and was used to stain GAS in immunofluorescence studies.

Adhesion assay. A human keratinocyte line derived from a well-differentiated squamous cell carcinoma (SCC-13), human soft palate keratinocytes (OKP7), and primary cultured neonatal foreskin keratinocytes (strain N) were described previously (13, 25). Cells were seeded into 24-well tissue culture plates (Costar Corp., Cambridge, MA) at $\sim 10^4$ cells per well in serum-free, antibiotic-free keratinocyte medium (GIBCO BRL, Gaithersburg, MD), supplemented with 0.1 ng/ml epidermal growth factor and 50 μg/ml bovine pituitary extract and grown to semiconfluence ($\sim 10^5$ cells or 75%). The monolayers were washed with warmed RPMI 1640 medium three times to remove unattached cells, then inoculated with $\sim 5 \times 10^6$ CFU of GAS in a final volume of 0.3 ml. The bacteria were sedimented onto the monolayers by centrifugation at 1,500 rpm for 8 min in a bench top clinical centrifuge. After incubation for 30 min at 37°C in 5% CO_2 and 100% relative humidity, the monolayers were washed five times with warm serum-free RPMI 1640 medium to remove unattached bacteria. To quantify bound bacteria, the cells were detached by adding 200 μl 0.25% trypsin/0.5 mM EDTA for 10 min at 37°C and then were lysed with 200 μl 0.02% Triton X-100 (final concentration 0.01%). The contents of each well were agitated for 15 s with a vortex mixer to evenly disperse the bacteria, and then were serially diluted in water, and spread on 5% sheep blood agar plates for quantitative culture. To confirm these results with an alternate method, the monolayers were fixed by heating to 75°C, stained with Gram's stain, and examined by light microscopy.

Immunofluorescent staining of cultured keratinocytes. Keratinocytes were cultured on 12-mm glass coverslips until semiconfluence. Monolayers were studied both for CD44 expression alone in the noninfected state or for simultaneous GAS binding and CD44 distribution. The cells were fixed with 2.5% glutaraldehyde in PBS, pH 7.4, for 3 h at room temperature or overnight at 4°C. Glutaraldehyde was removed by washing with PBS before incubating coverslips with PBS containing 0.5% BSA (0.5% BSA/PBS) for 30 min at room temperature to block nonspecific binding. Primary antibodies (anti-CD44 mAb IM7.8.1 at a final concentration 5 μg/ml and rabbit anti-GAS

serum at 7.5 μg/ml) were incubated with the cells in 0.5% BSA/PBS for 1 h at 4°C, before washing twice with PBS to remove unbound antibody and incubating with secondary antibodies, FITC-conjugated goat anti–rat IgG (Sigma Chemical Co., St. Louis, MO) at 5 μg/ml and Texas Red–conjugated goat anti–rabbit IgG at 7.5 μg/ml in 0.5% BSA/PBS for 1 h at 4°C. Cells were washed twice with PBS and mounted for fluorescent visualization with a Nikon Diaphot-TMD microscope (Nikon, Inc., Melville, NY).

Immunofluorescent staining of tissue sections. Paraffin-embedded tissue sections were deparaffinized by heating at 65°C for 1 h, followed by sequential duplicate washes in 100% xylene, 100% ethanol, 95% ethanol, 80% ethanol, and distilled water. Samples were incubated with 0.5% BSA/PBS for 1 h before staining as described for cell cultures. All incubations were performed at room temperature.

Attachment inhibition assays. Keratinocytes were preincubated with mAb IM7.8.1 (dialyzed hybridoma supernatant), or medium alone, for 30 min at room temperature. The remainder of the assay was performed as described in the adhesion assay. As controls, monolayers were incubated with a nonblocking, anti-CD44 mAb (4A4 or 8d8 at 10 μg/ml). Potential adverse effect of mAb on bacterial inoculum was controlled for by quantitative culture with and without mAb.

Transfected cell adhesion assay. K562 (American Type Culture Collection, Rockville, MD), a human erythroleukemia cell line lacking CD44 expression, grows in suspension and was maintained in DME containing 10% FCS. K562 cells were transfected by electroporation with pCEPCD44R1 and pCEPCD44R2, both of which were constructed in the expression vector pCEP4 (Invitrogen Corp., Carlsbad, CA) and express the CD44 isoforms CD44v8-10 (CD44R1) and CD44v10 (CD44R2), respectively (25a). Transfectants were cultured under hygromycin B selection (200 μg/ml) in DME supplemented with 10% FCS. CD44 surface expression was confirmed by FACS® analysis. For GAS adhesion assays, 10^5 cells in 100 μl DME/10% FCS were mixed with 10^6 CFU of GAS in 100 μl of PBS. Assay tubes were rotated end over end for 90 min at room temperature. 1 ml DME/10% FCS was added to the cell suspensions and the tubes were centrifuged for 1 min at 2,000 rpm in a microcentrifuge. The supernatants were discarded and the pellets were washed again as above. The supernatants were discarded and the pellets were resuspended in residual medium. A drop of cell suspension from each assay tube was placed on a glass slide and covered with a glass cover slip. The cells were examined for the presence of adherent bacteria by light microscopy at ×400. A cell was considered positive if at least one streptococcal chain was seen in direct contact with the plasma membrane. At least 40 cells were counted (for each experimental condition) and the results were expressed as the percentage of cells with attached bacteria.

Hyaluronate synthase assay. Cells from an exponential phase, liquid culture of GAS were collected by centrifugation, washed twice in 10 mM phosphate buffer, pH 7, containing 1 mM magnesium chloride. The cells were incubated at 37°C for 30 min in the same buffer containing 1 mM DTT, 25% sucrose, and 25 μg/ml mutanolysin (Sigma Chemical Co.). After washing twice in the same buffer, the protoplasts were lysed by resuspension in 10 mM phosphate buffer, pH 7, containing 1 mM magnesium chloride. An aliquot of cell lysate was incubated at 37°C for 30 min in a 100 μl assay mixture containing 70 μg of cell lysate protein, ~ 100,000 cpm of UDP-[^{14}C]glucuronic acid (New England Nuclear, Boston, MA), 5 mM UDP-*N*-acetylglucosamine, 2 mM DTT, 20 mM magnesium chloride, and 40 mM phosphate buffer, pH 7. High M_r [^{14}C]-hyaluronic acid was isolated from the assay mixture on a Sephadex G50 spin column (Pharmacia, Piscataway, NJ) and quantified by scintillation counting. A sample containing cell lysate previously inactivated by boiling was included in each set of assays as a negative control.

Results

Acapsular GAS exhibit enhanced adherence to human keratinocytes. We used an in vitro assay system to assess the influence of the hyaluronic acid capsule on attachment of GAS to keratinocytes, the predominant cell type of pharyngeal epithelium and external skin. Because wild-type GAS strains that express different amounts of capsule also may differ with respect to other surface molecules, we compared two isogenic acapsular mutant strains with the encapsulated wild-type GAS strains from which the mutants were derived. GAS were sedimented onto a monolayer of primary cultured keratinocytes. After incubation for 30 min, unbound bacteria were removed by washing, the keratinocytes were detached and lysed, and the number of adherent bacteria was determined by quantitative cultures of the lysates. For both M type 18 GAS and M type 24 GAS, greater numbers of adherent bacteria were recovered from keratinocytes inoculated with the acapsular mutant (M+C−) compared with the parent strain (M+C+, Fig. 1). The magnitude of the difference in adherence between encapsulated strains and isogenic acapsular mutants depended both on the bacterial strain and the target keratinocytes: the absence of capsule resulted in a twofold increase in attachment of type 24 GAS to soft palate keratinocytes and a 10-fold increase in attachment to skin keratinocytes (Fig. 1). For type 18 GAS, the acapsular mutant was 30-fold more adherent to soft palate and eightfold more adherent to skin keratinocytes than was the encapsulated parent strain. These results demonstrate that encapsulated strains of GAS adhere to human keratinocytes, but that the presence of the hyaluronic acid capsule reduces attachment to a variable extent depending on the GAS strain and keratinocyte type.

Type 6 and 24, but not type 18, M proteins mediate attachment of GAS to keratinocytes. The observation that encapsulated strains attached less well than acapsular mutants suggested that the capsule might prevent the interaction of one or more bacterial surface molecules with specific receptor(s) on keratinocytes. Since M protein has been shown to participate in adherence of an M type 6 strain of GAS to keratinocytes, we investigated the relationship between capsule and M protein in GAS adherence by comparing attachment to keratinocytes of GAS wild type strains (M+C+) to that of isogenic M protein mutants (M−C+), capsule mutants (M+C−), and mutants in both M protein and capsule (M−C−). The M protein–deficient mutant of an M type 6 strain exhibited a sixfold reduction in attachment to external skin keratinocytes compared with the isogenic wild-type strain (Fig. 1); however, attachment to soft palate keratinocytes was threefold greater for the M protein–deficient mutant compared with the type 6 parent strain. These results imply that the M6 protein serves as a ligand for attachment of GAS to external skin keratinocytes, but not for keratinocytes derived from the oropharynx. In general, M protein–deficient mutant strains of either type 18 or type 24 GAS attached at least as well as the respective wild-type strains to either soft palate or external skin keratinocytes (Fig. 1). However, attachment to either type of keratinocytes by a mutant strain expressing neither M protein nor capsule was reduced 5- to 14-fold compared with the isogenic M type 24 strain deficient only in capsule. The latter result implies that type 24 M protein can participate in attachment of GAS to keratinocytes, but that M protein–mediated adherence is prevented by the presence of the hyaluronic acid capsule in the wild-type strain. By contrast, there was no difference in attachment of the type 18 M protein–deficient, acapsular mutant compared with the isogenic acapsular strain expressing type 18 M protein. Therefore, type 18 M protein appears not to mediate adherence to

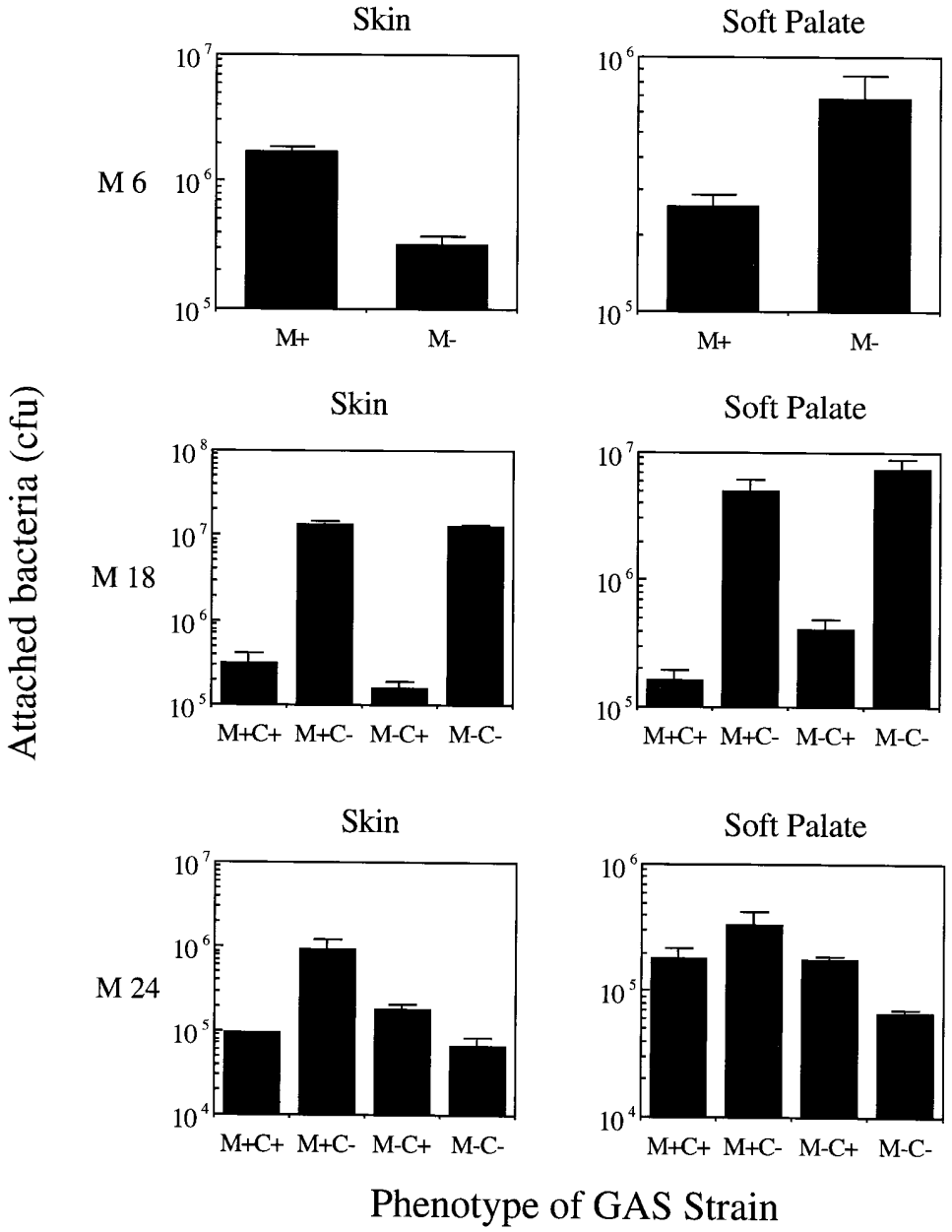
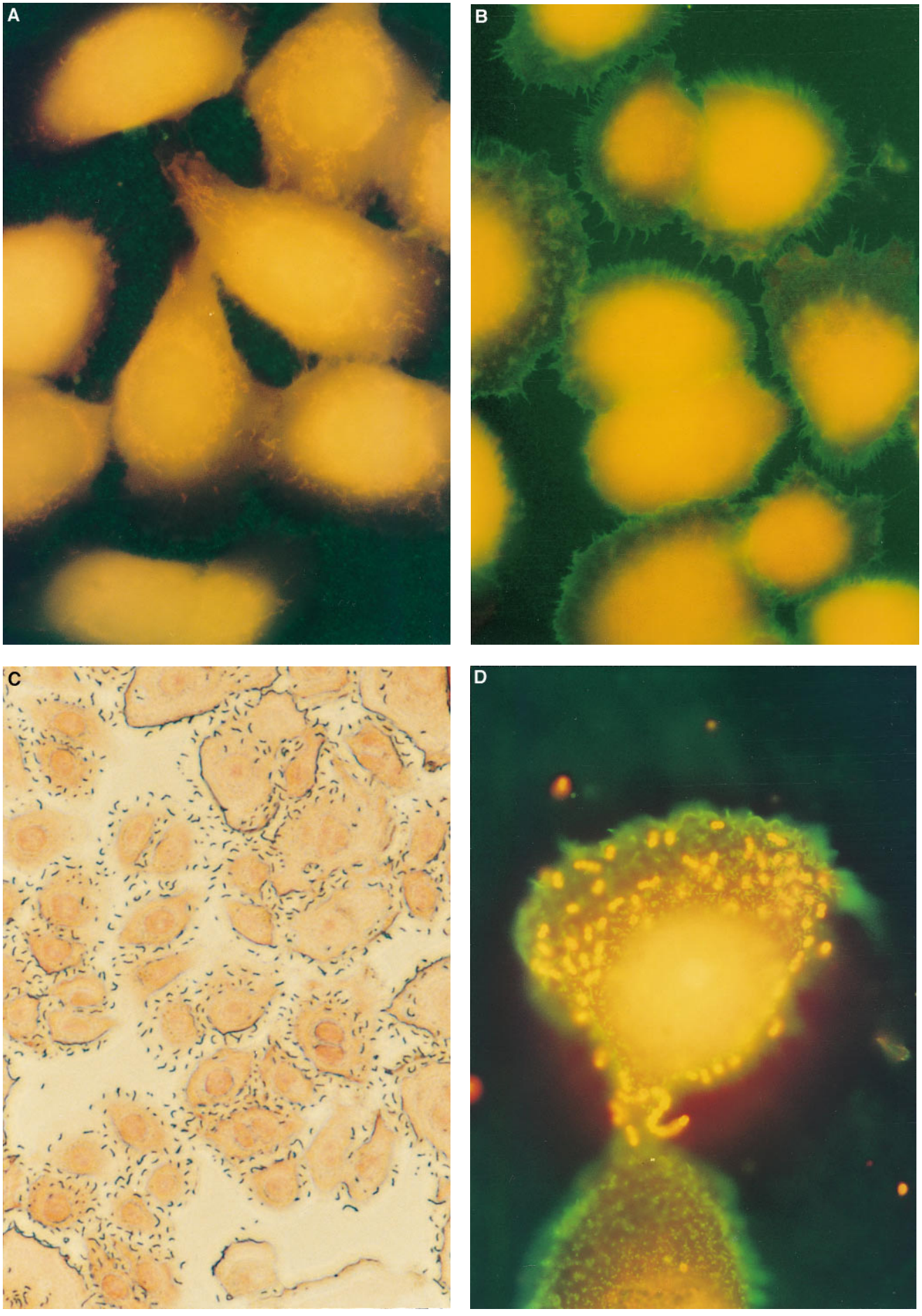


Figure 1. Effect of M protein and hyaluronic acid capsule on attachment of GAS to human soft palate and skin keratinocytes. Isogenic GAS mutants were derived from M type 6, 18, and 24 wild-type strains. M type of the parent strain for each group of strains is indicated on the left of the figure; M protein (*M*) and capsule (*C*) phenotype is represented by (+) or (−) which indicate presence or absence of the phenotype. The data are expressed as number of bacteria (in cfu) bound and represent means of duplicate or triplicate wells from a representative experiment.

keratinocytes, regardless of the presence or absence of capsule.

Hyaluronic acid capsule mediates attachment of GAS to keratinocytes. The experiments comparing attachment of M protein–deficient or capsule-deficient mutants of the type 24 strain of GAS revealed that attachment of the M protein–deficient, acapsular strain was reduced in comparison to the M protein–deficient, encapsulated strain (Fig. 1). Similar results were obtained in experiments examining attachment of the GAS strains to keratinocytes by direct microscopy: very few bacteria were seen attached to keratinocytes inoculated with the M protein–deficient, acapsular strain compared with keratinocytes inoculated with the isogenic M protein–deficient, capsule-positive strain (data not shown). These observations suggested that the hyaluronic acid capsule itself might mediate attachment, albeit less efficiently than type 24 M protein.

CD44 is the keratinocyte receptor for GAS hyaluronic acid capsule. CD44 is a major cell surface receptor for hyaluronic acid on many types of human cells such as lymphocytes, monocytes, and epithelial cells including keratinocytes. Since streptococcal hyaluronic acid appears to be structurally identical to mammalian hyaluronic acid, we tested the hypothesis that CD44 was the keratinocyte receptor for the GAS capsule. Incubation of keratinocytes from soft palate, external skin, or the SCC-13 squamous cell carcinoma line with monoclonal antibody to CD44 followed by FITC-labeled secondary antibody demonstrated surface expression of CD44 (Fig. 2). In similar experiments examining tissue sections of human palatine tonsil, we observed intense staining for CD44 localized to the cells lining the tonsillar crypts (not shown). The pattern of fluorescence of cultured keratinocytes indicated maximal expression of CD44 at the periphery of the cells, near intercellular junc-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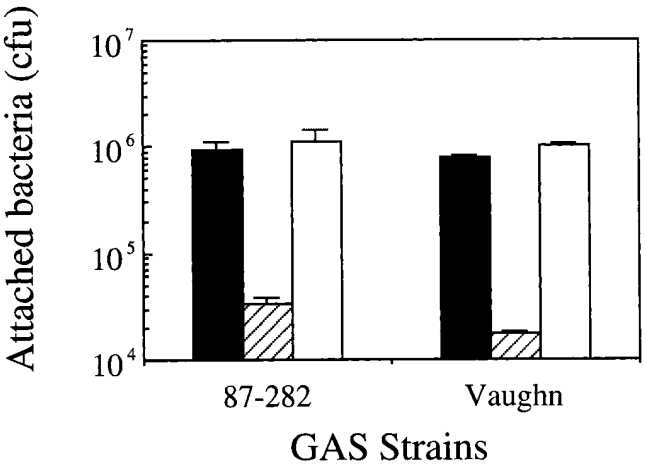

Figure 3. Inhibition of GAS attachment to soft palate keratinocytes by mAb to CD44. Bars indicate number of type 18 (*87-282*) or type 24 (*Vaughn*) GAS that bound after preincubation of the keratinocytes with buffer (*black bars*), with IM7.8.1, an anti-CD44 mAb that blocks hyaluronic acid binding (*hatched bars*), or with an anti-CD44 mAb (4A4 or 8d8) that does not block hyaluronic acid binding (*white bars*). The data represent means of triplicate wells of a representative experiment.

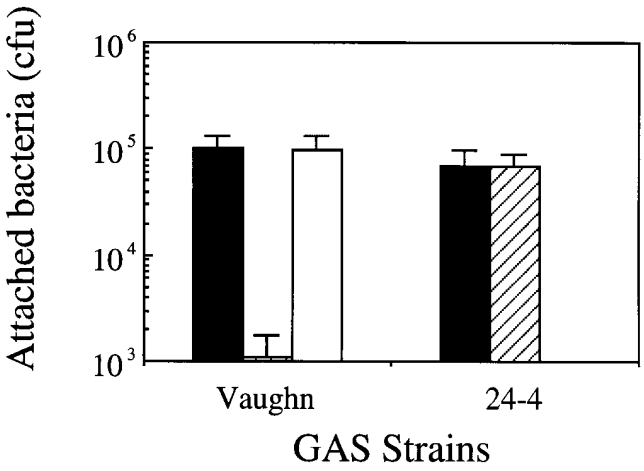

Figure 4. Specificity of CD44-mediated adherence for the hyaluronic acid capsule. Bars indicate the number of type 24 wild-type GAS (*Vaughn*) or acapsular mutant strain 24-4 GAS that bound to strain N primary skin keratinocytes after preincubation of the keratinocytes with buffer (*black bars*), with IM7.8.1, an anti-CD44 mAb that blocks hyaluronic acid binding (*hatched bars*), or with 8d8, an anti-CD44 mAb that does not block hyaluronic acid binding (*white bar*). Data represent means of triplicate wells.

tions with adjacent cells, and on the filopodia of nonconfluent cells. This distribution of CD44 as evidenced by immunofluorescence was very similar to the pattern of binding observed by direct microscopy of encapsulated GAS bound to keratinocytes (Fig. 2).

To determine whether CD44 was a receptor for GAS, we measured the ability of monoclonal antibodies to CD44 to block attachment of GAS to keratinocytes. Monoclonal antibodies 8d8 and 4A4 bind to CD44 on human keratinocytes, but do not prevent simultaneous binding of hyaluronic acid, while monoclonal antibody IM7.8.1 blocks hyaluronic acid binding to CD44. Preincubation of keratinocytes with the blocking antibody IM7.8.1 reduced attachment of the encapsulated type 18 strain 87-282 or of the type 24 strain Vaughn to soft palate or external skin keratinocytes by more than 90%, compared with attachment of the same GAS strains to untreated keratinocytes or to keratinocytes treated with a nonblocking antibody to CD44 (Fig. 3). As expected, preincubation of keratinocytes with IM7.8.1 had no effect on attachment of the acapsular mutant of strain Vaughn, 24-4 (Fig. 4).

Although the amount of capsule produced by individual strains varies from undetectable to abundant, nearly all GAS strains appear to contain the *has* gene cluster required for hyaluronic acid synthesis (12, 26). Therefore, it seemed likely that CD44 might serve as a receptor for attachment of diverse strains of GAS to human epithelial cells. This hypothesis was supported by results of experiments examining the capacity of IM7.8.1 to block attachment of other strains of GAS representing various M types and including strains in which capsule production was below the limits of detection in our assay. Hyaluronate synthase activity could be detected in each of these poorly encapsulated GAS strains using a sensitive assay for incorporation of [^{14}C]glucuronic acid from UDP-[^{14}C]glucuronic acid into high M_r hyaluronic acid, albeit at levels only 10–20% of that in highly encapsulated strains (data not shown). In all cases, preincubation of keratinocytes with IM7.8.1 reduced attachment of GAS by $\geq$ 84% (Table II).

Transfection of human cells with CD44 confers the ability to bind GAS. The finding that a mAb specific for CD44 blocked binding of GAS to keratinocytes is evidence that the interaction between the streptococcal capsule and host cell CD44 participates in the attachment of GAS to epithelial cells. To test whether this interaction could mediate attachment to cells that otherwise do not bind GAS, K562 erythroleukemia cells were transfected with cDNA encoding either of two CD44 isoforms normally expressed on human keratinocytes, CD44v8–10 and CD44v10. Control or transfected K562 cells were incubated in suspension with GAS, then scored for GAS binding after removal of unbound bacteria by differential centrifugation. By microscopy, GAS were observed in close association with K562 cells expressing CD44, but not with untransfected control cells. For the six wild-type strains of GAS

Figure 2. Distribution of CD44 and pattern of attachment of GAS on human keratinocytes. (*A*) SCC-13 keratinocytes stained with FITC-labeled secondary antibody without prior incubation with primary antibody. (*B*) SCC-13 keratinocytes stained with FITC-labeled secondary antibody after incubation with anti-CD44 mAb IM7.8.1, demonstrating intense green fluorescence at the periphery of the cells. (*C*) Gram's stain demonstrating binding of GAS strain Vaughn to the periphery of OKP7 keratinocytes. (*D*) Dual immunofluorescence microscopy showing pattern of binding of GAS strain 940771 (stained with anti–group A carbohydrate and Texas Red–labeled secondary antibody) to SCC-13 keratinocytes (stained with anti-CD44 mAb and FITC-labeled secondary antibody).

Table II. Monoclonal Antibody Inhibition of GAS Attachment to Human Keratinocytes

Strain	M type	Capsular HA‡ (fg/CFU)	Antibody treatment*		Percent inhibition
			None	IM7.8.1	
Vaughn	24	70	124000±3800	13740±4000	89
94467	3	49	63000±13000	4300±2500	93
DLS003	3	< 10	320000±56000	6200±2500	98
950358	1	< 10	181000±15500	29000±4800	84
SS-644	27	< 10	3920000±1200000	220000±100000	94

*Values represent CFU of GAS attached to SCC-13 keratinocytes in the presence or absence of anti-CD44 monoclonal antibody IM7.8.1. Data represent means and SD of triplicate wells. ‡Cell-associated hyaluronic acid.

tested, binding was observed to 52–78% of K562 cells expressing either isoform of CD44 (Table III).

To prove that attachment to CD44 was mediated by the hyaluronic acid capsule even in strains that produced very small amounts of hyaluronic acid, we constructed a targeted knockout of *hasA*, the hyaluronate synthase gene, in one such strain, DLS003. As expected, no binding was observed for the capsule-negative mutant strain 003ΔhasA (Table III). Similarly, no binding was observed for TX72 or 24-4, capsule-negative mutants of the highly encapsulated strains 87-282 and Vaughn, respectively. These experiments established that CD44 mediated attachment of GAS to transfected K562 cells. They further demonstrated that the hyaluronic acid capsule, even in poorly encapsulated strains, is the bacterial ligand that binds to CD44.

Discussion

Our results comparing attachment of isogenic acapsular mutant strains confirmed indirect evidence from earlier studies that the hyaluronic acid capsule decreases the overall adhesiveness of GAS to epithelial cells. Since GAS infection begins with colonization of the throat or skin, production of a hyaluronic acid capsule might be viewed as disadvantageous by virtue of the reduced adherence of encapsulated bacteria to epithelial cells. However, the effect of the hyaluronic acid capsule to reduce overall adhesiveness of GAS must be reconciled with observations that capsule expression enhances pharyngeal colonization by GAS in vivo. In two previous studies, capsule-deficient mutant strains of GAS were impaired in their ability to colonize the pharynx in mice (12, 14). Since capsule-deficient strains are susceptible to complement-mediated phagocytic killing, their decreased ability to colonize may reflect more efficient clearance by host phagocytes. Our experiments examining the roles of the hyaluronic acid capsule and M protein in attachment of GAS to cultured human keratinocytes offer an alternative, but not mutually exclusive, explanation for the enhanced ability of encapsulated GAS to colonize the pharynx: the hyaluronic acid capsule mediates attachment of GAS to pharyngeal and epidermal keratinocytes via the host hyaluronic acid-binding molecule, CD44. Remarkably, attachment of nonmucoid or poorly encapsulated isolates was also inhibited by ~ 90% with an anti-CD44 mAb indicating that hyaluronic acid–mediated attachment occurred not only in highly encapsulated GAS strains, but also in strains that express nominal amounts of capsule. These results were confirmed in experiments with CD44-transfected K562 cells that demonstrated hyaluronic acid–mediated attachment of GAS to CD44-positive cells.

Previous studies have identified several GAS surface molecules that may participate in bacterial attachment to one or more types of mammalian cells. These candidate adhesins include lipoteichoic acid (27), M protein (3, 9, 28), fibronectin-binding proteins (2, 4, 5), and glyceraldehyde-3-phosphate dehydrogenase (29). Because the hyaluronic acid capsule forms an outermost layer on GAS cells, the capsule may modulate or prevent interaction of bacterial cell wall components or surface proteins with host cell receptors. In this study, for example, expression of type 24 M protein did not affect attachment of a highly encapsulated strain, but did increase binding of capsule-deficient mutants to both skin and soft palate keratinocytes, indicating that abundant capsule expression by mucoid strains of GAS may prevent M protein–mediated adherence. These results are consistent with those of Courtney et al. (30) who found that M protein–mediated attachment of type 24 GAS to HEp-2 cells was inhibited by the presence of capsule. Our results suggest also that the function of M protein in adherence varies among different serotypes. Using the same

Table III. GAS Attachment to K562 Cells Expressing One of Two Major Keratinocyte Isoforms of CD44

Strain	M type	Source of isolate	Capsular HA‡ (fg/CFU)	CD44 isoform expressed by target host cells*		
				None	CD44v8-10	CD44v10
87-282	18	ARF	70	0	58	63
TX72	18	(87-282,cap-)	< 10	0	0	0
Vaughn	24	ARF	65	0	68	59
24-4	24	(T24,cap-)	< 10	0	0	0
950771	3	NF	41	0	77	78
DLS003	3	NF	< 10	0	77	76
003ΔhasA	3	(DLS003,cap-)	< 10	0	0	0
950802	1	NF	< 10	0	56	52
JRS4	6	REF	< 10	0	58	ND

*Data represent the percentage of host cells with adherent bacteria and are means of duplicate experiments. At least 40 cells were counted for each determination. ‡Cell-associated hyaluronic acid. *ARF*, acute rheumatic fever; *cap-*, unencapsulated mutant; *NF*, necrotizing fasciitis; *REF*, laboratory and reference strain.

wild-type and M protein–deficient strains as Okada et al. (3), we confirmed their finding that M protein enhanced the attachment of the M6 strain JRS4 to skin keratinocytes. However, expression of the M6 protein did not increase attachment to soft palate keratinocytes. In contrast to types 6 and 24, the type 18 M protein did not appear to play a significant role in adherence. An acapsular, M protein–deficient mutant attached as well to skin or soft palate keratinocytes as the acapsular, type 18 M protein–positive strain, suggesting that M protein is unnecessary for adhesion of type 18 strains. Thus, although M protein can function as an adhesin, its role in GAS attachment depends upon M type, the masking effect of capsule, and characteristics of the target epithelial cell.

Most pharyngeal isolates of GAS grow as nonmucoid or glossy colonies on solid media; such strains generally have been considered not to produce capsule. In this investigation, however, mAb to CD44 blocked attachment not only of strains that produced measurable amounts of hyaluronic acid capsule, but also of strains in which the level of capsule production was below the limit of detection in our assay. Furthermore, CD44-mediated binding of such a minimally encapsulated strain could be prevented by inactivating *hasA*, the gene encoding hyaluronate synthase. These results suggest that many GAS strains previously considered acapsular on the basis of colony morphology may produce amounts of capsular hyaluronic acid sufficient to mediate attachment to CD44 on epithelial cells. Although GAS strains differ in the quantity of capsule they produce, the structure of the capsular polysaccharide appears to be invariant. The invariant structure and surface exposure are distinctive features of the GAS capsule in comparison to M protein, fibronectin-binding proteins, or cell wall components such as lipoteichoic acid whose role in adherence is affected by whether or not a surface protein is expressed, allelic variation in protein structure, and accessibility of the potential ligand(s) on the bacterial surface. Therefore, the hyaluronic acid capsule may function as a universal adhesin for most GAS strains associated with human infection, permitting attachment of the bacteria to epithelial cells by a mechanism that is independent of strain or serotype-specific expression of alternative ligands.

The cellular receptor for the GAS capsule, CD44, is a transmembrane glycoprotein expressed on the surface of many epithelial, mesenchymal, and hematopoietic cells. In addition to the standard or hematopoietic form, CD44 occurs in a variety of variant isoforms that arise from alternative splicing of at least 10 genomic exons in addition to those encoding standard CD44; CD44v8-10, CD44v10, and CD44v3-10 are major variants on human skin keratinocytes, where CD44 molecules are localized primarily on filopodia of cultured cells and at intercellular junctions in tissue sections (31, 32). CD44 is expressed throughout the epidermis from the germinative basal layer to the granular layer just below the stratum corneum (33, 34). CD44 expression is upregulated during inflammation, injury, and healing (35, 36). Thus, minor trauma that frequently precedes streptococcal skin infection may serve not only to breach the stratum corneum, but also to upregulate receptors for attachment of GAS on the injured epidermis. The stratified squamous epithelium of the pharynx, in contrast to that of external skin, is not protected by a cornified layer. In immunofluorescence studies of histologic sections of human tonsils, we found that CD44 is expressed both on the epithelial surface of the tonsillar crypts and on the pharyngeal aspect of the tonsils. The greater exposure of CD44-positive cells in the pharyngeal epithelium may account in part for the higher rate of GAS colonization of the pharynx compared with external skin where CD44-bearing keratinocytes are covered by the stratum corneum.

The ubiquity of GAS infections is evidence of the successful adaptation of this organism to survival within the human host. Results of this investigation define CD44 as a widely distributed receptor for attachment of GAS to the epithelial tissues where colonization and infection occur. The demonstration that this binding interaction mediates attachment of diverse strains of GAS regardless of serotype or the presence of alternative adhesins suggests that CD44 represents a major cellular receptor for GAS on human epithelial cells.

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