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Research Article

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Hyaluronic Acid Capsule and the Role of Streptococcal Entry into Keratinocytes in Invasive Skin Infection

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Abstract

It has been suggested that entry of pathogenic bacteria, including streptococci, into epithelial cells may represent an early stage of invasive infections. We found that poorly encapsulated wild-type strains and unencapsulated mutants of group A *Streptococcus* entered cultured human keratinocytes with high efficiency, while strains that produced large amounts of hyaluronic acid capsule did not, regardless of M-protein type or clinical source of the isolate. However, encapsulated streptococci produced extensive local necrosis and systemic infection in a mouse model of skin infection, while an isogenic acapsular strain did not. The results implicate the hyaluronic acid capsule as a virulence factor in soft tissue infection. Entry of poorly encapsulated group A *Streptococcus* into human epithelial cells does not appear to represent an initial step in invasive disease; rather, the capacity of encapsulated strains to avoid uptake by epithelial cells is associated with enhanced virulence in skin and soft tissue infection. (*J. Clin. Invest.* 1996. 98:1954–1958.) Key words: keratinocyte • group A *Streptococcus* • hyaluronic acid • virulence • capsular polysaccharide

Introduction

Streptococcus pyogenes or group A *Streptococcus* (GAS),[1] the agent of streptococcal sore throat and external skin infections, occasionally causes life-threatening infections such as necrotizing fasciitis in which the bacteria spread rapidly through deeper soft tissues (1, 2). Although GAS generally has been considered an extracellular pathogen, two recent studies showed that GAS entered epithelial carcinoma cells (3, 4). Entry into host cells is critical for survival of bacterial pathogens that replicate in an intracellular milieu (5, 6). For organisms that replicate at extracellular sites, however, the significance of bacterial entry into host cells is less well defined. It has been suggested that entry into epithelial cells represents an early step of invasive infection (4, 7). Bacteria that gain access to the intracellular compartment may be protected from the actions of complement proteins, specific antibodies, and phagocytic leukocytes. If GAS can survive and proliferate inside host epithelial cells, these cells could provide an important sanctuary site from which invasive infection develops.

The initial interaction of GAS with the human host is at the epithelial surfaces of the pharynx or external skin. At both anatomic sites, the major cell type that forms the stratified squamous epithelium is the keratinocyte. The goal of this study was to investigate whether GAS enter normal human keratinocytes, and, if so, to determine the importance of the process in pathogenesis of streptococcal skin and soft tissue infection.

The hyaluronic acid capsule of GAS has been shown to enhance virulence in systemic and intranasal challenge models (8–11). However, the role of the capsule in pathogenesis of skin and soft tissue infections has not been studied in detail. A second objective, therefore, was to determine the effect of the capsule on GAS entry into keratinocytes and on invasive soft tissue infection.

Methods

Bacteria. 24 GAS clinical isolates (Table I) used in this study were provided by Drs. R. Facklam (CDC, Atlanta, GA), E. Kaplan (Minneapolis, MN), and D. L. Stevens (Boise, ID) or were from the Channing Laboratory collection. GAS strain TX72 is an unencapsulated isogenic mutant derived by Tn*916* mutagenesis from the mucoid M-type 18 GAS strain 282 (87-282) (11); strains 24-4 and 24-72 are unencapsulated mutants derived from the M-type 24 GAS strain T24 (strain Vaughn) (11). For internalization assays, GAS were grown in Todd-Hewitt broth to exponential-phase, sedimented by centrifugation, and resuspended in tissue culture medium before addition to keratinocytes.

Capsular hyaluronic acid measurement. To determine the amount of hyaluronic acid capsule produced by each GAS strain, cells from a 10-ml exponential-phase culture were washed twice with water, then suspended in 0.5 ml water. Capsule was released by shaking with 1 ml of chloroform. After clarifying the sample by centrifugation, the hyaluronic acid content of the aqueous phase was determined by measuring absorbance at 640 nm after adding to the sample 2 ml of a solution containing 20 mg of 1-ethyl-2-[3-(1-ethylnaphtho-[1,2-*d*]thiazolin-2-ylidene)-2-methylpropenyl]naptho-[1,2-*d*]thiazolium bromide (Stains-all; Sigma Chemical Co., St. Louis, MO) and 60 μl of glacial acetic acid in 100 ml of 50% formamide. Absorbance values were compared with a standard curve generated using known concentrations of hyaluronic acid (12, 13).

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

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Table I. Internalization of GAS by Human Keratinocytes (SCC-15)

Strain	M-type	Clinical source of isolate	Capsular HA	Intracellular bacteria
			fg/CFU	*mean CFU**
9003	18	ARF	77	12
282	18	ARF	75	12
SS109	30	Unknown/reference	70	22
T24	24	ARF	69	25
DLS030	18	Bacteremia	63	17
SS901	31	Unknown/reference	55	100
94467	3	NF	49	300
SS90	3	Unknown/reference	45	188
94421	3	NF+TSS	43	95
950771	1	NF	41	400
950532	3	NF	39	430
T6	6	Unknown/reference	32	118
950802	3	NF+TSS	< 10	8600
346	18	Pharyngitis	< 10	5220
764	3	ARF	< 10	3490
136	3	ARF contact	< 10	16000
DLS003	3	NF	< 10	3260
DLS028	4	NF+TSS	< 10	2980
DLS049	1	TSS (postpartum)	< 10	6540
SS644	27	Unknown/reference	< 10	256000
SS894	30	Unknown/reference	< 10	81100
SS288	4	Unknown/reference	< 10	166300
SS1170	8	Unknown/reference	< 10	158000
JRS4	6	Unknown/reference	< 10	31400

GAS strains are ranked in the table according to the amount of cell-associated hyaluronic acid capsule produced. *ARF*, acute rheumatic fever; *HA*, hyaluronic acid; *NF*, necrotizing fasciitis; *TSS*, toxic shock syndrome. *Mean of triplicate determinations.

Internalization assay. An established human keratinocyte cell line (SCC-15), originally derived from a well-differentiated, squamous cell carcinoma of the ventral tongue (14), was grown to semiconfluence ($\sim 10^5$ cells/well) in 24-well tissue culture plates (Costar Corp., Cambridge, MA) in serum-free, antibiotic-free keratinocyte medium (Gibco Laboratories, Grand Island, NY) supplemented with 0.1 ng/ml epidermal growth factor and 50 μg/ml bovine pituitary extract. The assay for internalization of GAS was a modification of that described by Rubens et al. (7). The monolayers were washed three times with sterile PBS, pH 7.4, to remove unattached cells, then inoculated with $\sim$ 100,000 CFU of GAS. The bacteria were sedimented onto the cells by centrifugation at 500 *g* for 6 min. After incubation for 3 h at 37°C in 5% CO_2, the cells were washed with PBS, then fresh medium containing gentamicin (200 μg/ml) was added to kill the remaining extracellular bacteria. 45 min later, a 50-μl aliquot of supernatant fluid was removed for quantitative culture to confirm killing of extracellular bacteria. To recover intracellular bacteria, the antibiotic-containing medium was removed (three to four washes), the cells were detached by incubation with 200 μl 0.25% trypsin/0.5 mM EDTA for 10–12 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 plated on 5% sheep blood agar plates for quantitative culture. Selected experiments were repeated using OKP7 cultured soft palate keratinocytes (15), and strain N neonatal foreskin keratinocytes (16).

Electron microscopy. Strain N foreskin keratinocyte monolayers inoculated as described above, were washed, and then fixed with glutaraldehyde. The monolayers were processed for transmission electron microscopy as described (17).

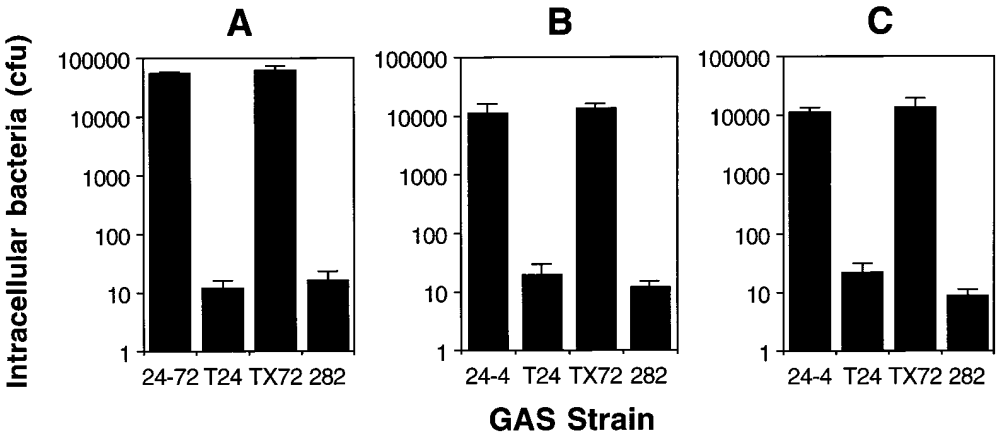
Recovery of internalized bacteria over time. SCC-15 monolayers were prepared and inoculated with GAS strain 24-72 as in the internalization assay, except that the inoculation period was limited to 1 h, after which the monolayers were washed, and fresh medium containing gentamicin was added. At 2, 6, 12, or 24 h after initial inoculation the antibiotic-containing medium was washed away, the keratinocytes were lysed, and intracellular bacteria were recovered as described in the internalization assay. Cytotoxicity was estimated at each time point by assessing the ability of keratinocytes from replicate wells to exclude trypan blue.

Mouse model of invasive skin and soft tissue infection. Fur was removed from a 2 × 2 cm area over the left flank of adult CD-1 mice (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) by applying a depilatory agent (Nair; Carter Products, New York) for 5 min and then wiping clean with cotton swabs. Then the area was prepared with 70% ethanol and inoculated by injecting 50 μl of a suspension (~ 5–10×10^6 CFU) of either an unencapsulated mutant (24-4) or the encapsulated parent GAS strain (T24) using a 27-gauge needle so that a superficial bleb was raised immediately below the skin surface. Bacteremia was assessed daily by culturing two or three drops of tail vein blood on blood agar plates. The animals were killed 7 d after inoculation and the skin surrounding the inoculation site was excised. One half the specimen was placed into formalin for histopathology. The other was trimmed to $\sim$ 100 mg, then homogenized in equal parts Todd-Hewitt broth and 0.025% Triton X-100 with mortar and pestle (Wheaton, Millville, NJ) and plated for quantitative culture.

Results

Internalization of GAS strains by human keratinocytes. We examined GAS strains of various M-protein types and degrees of encapsulation in an assay measuring internalization of the bacteria by cultured human keratinocytes, the major cell type of pharyngeal epithelium and external skin. Comparable numbers of organisms were used for each strain in the internalization assays, so the number of intracellular organisms recovered in the assays represents a direct measure of internalization efficiency. The efficiency of bacterial entry into keratinocytes varied widely among strains, although the behavior of each individual strain was quite reproducible. Efficiency of cell entry was not correlated with clinical source of the isolate nor with M-protein type (Table I). However, there was a striking inverse relationship between hyaluronic acid capsule expression and efficiency of GAS entry into keratinocytes. We recovered 3,000–250,000 intracellular CFU of GAS strains that produced little or no capsule, but only 12–400 CFU of the encapsulated strains. Thus, GAS strains that produced measurable amounts of capsular hyaluronic acid had markedly lower internalization efficiency than poorly encapsulated strains.

Internalization of isogenic strains. To investigate directly the effect of the hyaluronic acid capsule on internalization of GAS by keratinocytes, we compared in the same assay isogenic unencapsulated mutants of M-type 18 or M-type 24 GAS with their respective encapsulated parent strains (11). The unencapsulated mutants showed 5,000-fold greater internalization efficiencies than the corresponding encapsulated parent strains in SCC-15 cells (Fig. 1). To be certain the results obtained using keratinocytes derived from a carcinoma cell line were representative of those for normal human keratinocytes, the same studies were repeated using primary cultured keratinocytes derived from normal human external skin



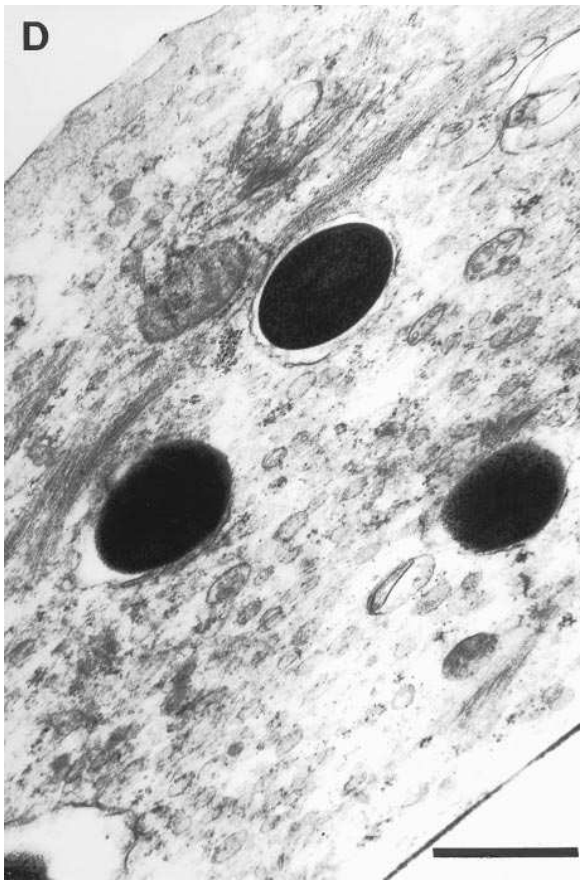


Figure 1. Internalization of encapsulated or isogenic unencapsulated GAS strains by human keratinocytes cultured from an oral squamous carcinoma (SCC-15, *A*), normal soft palate (OKP7, *B*), or normal foreskin (strain N, *C*). The three graphs illustrate that the unencapsulated strains (TX72 and 24-4) enter keratinocytes in 1,000- to 5,000-fold greater numbers than the respective parent strains (282 and T24) ($P < 0.01$, for comparisons between unencapsulated mutant and encapsulated parent strain for both pairs of isogenic strains for all three cell lines, two-tailed *t* test). *D* is a transmission electron micrograph showing a section of a strain N foreskin keratinocyte inoculated with unencapsulated GAS strain TX72. Three intracellular GAS organisms are seen as electron dense, ovoid structures, each surrounded by a vacuolar membrane. The apical surface of the keratinocyte is at the upper left, the basal surface at the lower right (bar, 1 μm).

(strain N) or normal human oropharynx (OKP7). Similar results were obtained for all three keratinocyte lines (Fig. 1).

Unencapsulated GAS were visualized by electron microscopy in membrane-bound vacuoles within keratinocytes (Fig. 1 *D*); no intracellular bacteria were identified in comparable images of keratinocytes inoculated with encapsulated GAS. Control experiments demonstrated little or no difference between unencapsulated and encapsulated strains in growth rate, chain length, aggregation, or antibiotic susceptibility in the conditions used in the assays (data not shown). Therefore, none of these potential artifacts account for the dramatic differences in internalization efficiency between unencapsulated and encapsulated strains. Furthermore, no viable bacteria were recovered from control wells without keratinocytes, from samples of supernatants after gentamicin exposure, or from wells in which the keratinocytes were lysed in the presence of gentamicin.

Time course of recovery of intracellular GAS. To investigate the fate of internalized GAS, we studied recovery over time of intracellular bacteria from infected keratinocytes continuously maintained in the presence of gentamicin. By 6 h after inoculation, the number of viable intracellular bacteria had declined to 17% of the number of intracellular organisms recovered 2 h after inoculation (Fig. 2). The number of intracellular GAS continued to fall over the next 18 h to $\sim$ 1% of the 2-h value. At 24 h after inoculation as many as 50% of the ke-

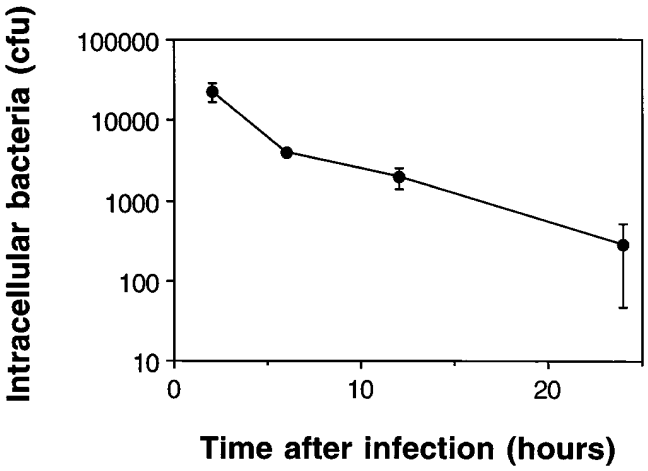


Figure 2. Survival of GAS within keratinocytes. After inoculation of SCC-15 cells with GAS strain 24-72, the cells were incubated in the presence of gentamicin for various periods, then washed and lysed for quantitative culture. The data represent means±SD of triplicate wells.

ratinocytes in the infected cultures appeared nonviable by morphology and failure to exclude trypan blue, indicating that keratinocyte death accounted for some, but not all, of the decline in number of intracellular bacteria. Thus, internalized GAS failed to multiply within keratinocytes and at least 99% of the intracellular bacteria became nonviable within 24 h.

Effect of hyaluronic acid capsule in a mouse model of invasive skin and soft tissue infection. The results of the experiments described above present an apparent paradox: poorly encapsulated GAS, regardless of clinical source, entered keratinocytes with high efficiency, while encapsulated GAS did not. Yet, encapsulation has been associated with GAS virulence in epidemiologic studies and in experimental models of systemic infection and upper airway colonization (8, 11, 18). If entry of GAS into keratinocytes were a virulence mechanism for skin and soft tissue infection, unencapsulated GAS would be expected to be virulent in a skin and soft tissue infection model, since such strains enter keratinocytes at high efficiency. If, however, entry of GAS into keratinocytes were not a virulence mechanism, but rather represented a failure of the bacteria to avoid ingestion by host cells, unencapsulated GAS would be expected to exhibit reduced virulence. To test directly the effect of the hyaluronic acid capsule on invasive skin and soft tissue infection, we compared encapsulated type 24 GAS with an isogenic unencapsulated mutant in a mouse model of cutaneous bacterial challenge. Bacteria were introduced by injection just below the skin surface to simulate cutaneous inoculation of GAS by minor trauma. This model differs somewhat from the air sac model used by Boyle and co-workers (19, 20) in that the inoculum is injected very superficially, in a small volume (50 μl), and with minimal trauma to the tissues. The majority of animals inoculated with the encapsulated strain developed bacteremia, while none challenged with the unencapsulated strain had evidence of systemic infection (Table II). All 10 animals challenged with the encapsulated strain developed necrotic local skin lesions with underlying abscesses, while only 3 of 11 mice challenged with the unencapsu-

Table II. Effect of GAS Capsule on Virulence in a Mouse Model of Skin and Soft Tissue Infection: Size of Skin Lesion and Incidence of Bacteremia after Cutaneous Inoculation with Encapsulated or Unencapsulated GAS

	T24 (Encapsulated strain)		24-4 (Unencapsulated strain)	
Day*	Mean lesion diameter±SD (mm)‡	No. of mice with positive blood cultures	Mean lesion diameter±SD (mm)‡	No. of mice with positive blood cultures
1	6.6±1.6	2/10	3.0±0.9	0/10§
2	7.2±1.3	5/10	3.3±1.0	0/10§
3	6.9±1.3	3/10	3.0±1.8	0/11
4	5.9±1.6	7/10	1.5±1.8	0/10§
5	5.8±1.8	5/10	1.9±1.7	0/11
6	6.9±2.0	8/10	1.3±1.8	0/11
7	7.2±1.6	5/10	1.9±2.3	0/11

Both mean lesion diameter and incidence of bacteremia were significantly greater for animals challenged with wild-type compared with unencapsulated GAS on each of the 7 d after inoculation. ($P < 0.01$ for both comparisons on all days, two-tailed t test.) §Blood culture not obtained from one animal. ‡Mean diameter of skin lesion at inoculation site. *Number of days after inoculation.

Table III. Effect of GAS Capsule on Virulence in a Mouse Model of Skin and Soft Tissue Infection: Gross Appearance, Histopathology and Quantitative Culture of Inoculation Site 7 d after Inoculation with Encapsulated (T24, n = 10) or Unencapsulated (24-4, n = 11) GAS

Strain	No. of mice	Gross appearance	Histopathology	Culture of lesion
				CFU/gram
T24	10	Indurated with purulent mass and necrotic eschar	Dermal necrosis and subcutaneous abscess	$\geq 10^5$
24-4	2	Indurated with purulent cystic lesion	Dermal necrosis and subcutaneous abscess	$\geq 10^5$
24-4	1	Indurated with purulent eschar	Purulent dermatitis	$\sim 10^4$
24-4	2	Indurated plaque	Mild dermal and subcutaneous inflammation	$\sim 10^4$
24-4	6	No lesion	No significant abnormality	0

lated strain had similar lesions 7 d after challenge (Table III). Based on these results, we conclude that the hyaluronic acid capsule enhances the capacity of GAS to produce invasive soft tissue infection and systemic dissemination after introduction of the organisms into the skin.

Discussion

Many bacteria pathogenic for humans have been shown to enter eukaryotic cells in culture (5, 6). For bacteria that grow within cells during infection, entry into appropriate target cells of the host represents an important step in establishing infection. However, the results of this study indicate that bacterial entry into epithelial cells is not necessarily associated with invasive infection. Strains of GAS that entered keratinocytes with high efficiency were those that lack hyaluronic acid capsules. Once inside the keratinocytes, the organisms did not proliferate, but rather exhibited a steady decline in viability over the 24 h after cell entry. Greco et al. (3) observed a similar decline over time in recovery of viable GAS from HeLa or HEp2 cells. These observations suggested that GAS entry into epithelial cells might not represent a virulence mechanism, a conclusion that was supported by comparing encapsulated and unencapsulated GAS in a skin infection model. The encapsulated strain (that entered keratinocytes poorly) was highly virulent, producing extensive local tissue necrosis and secondary bacteremia, while the unencapsulated strain (that entered keratinocytes at high efficiency) produced fewer and less severe local lesions, and no animals developed bacteremia.

Our data support an alternative hypothesis for the role of cellular entry of GAS and perhaps other pathogens that grow in an extracellular milieu within the infected host: internalization of GAS by keratinocytes does not promote subsequent invasive infection, but rather represents a failure of the bacteria to avoid ingestion by host cells. It remains to be determined whether internalization of GAS by epithelial cells contributes in a meaningful way to the successful containment of infection by inhibiting the growth of intracellular bacteria or by sloughing of infected cells from the mucosal or external skin surface. Previous studies of encapsulated GAS showed that the hyaluronic acid capsule rendered the bacteria resistant to opsonophagocytic killing by human blood leukocytes by preventing access of phagocyte complement receptors to C3b bound to the bacterial cell (8, 21). The present investigation indicates that the capsule also prevents internalization of GAS by epithelial cells, perhaps by blocking contact between receptors on the epithelial cells and their cognate ligands on the bacterial surface. It is likely that blocking effect of the hyaluronic acid capsule on internalization of GAS is not limited to keratinocytes but also modulates interactions of the bacteria with other types of epithelial cells. LaPenta et al. (4) found that stationary phase GAS were internalized by A549 lung carcinoma cells with much greater efficiency than exponential phase cells, a difference that could be accounted for by the difference in capsule expression between stationary phase and exponential phase GAS. Thus, the GAS capsular polysaccharide acts as a virulence factor by preventing ingestion of the bacteria not only by phagocytic blood leukocytes but also by epithelial cells. That highly encapsulated GAS strains resist internalization by host cells enhances the capacity of such strains to invade soft tissues by an extracellular route, leading to local tissue necrosis and systemic infection.

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References

1. Bisno, A.L., and D.L. Stevens. 1996. Streptococcal infections of skin and soft tissues. *N. Engl. J. Med.* 334:240–245.

2. Stevens, D.L. 1992. Invasive group A streptococcus infections. *Clin. Infect. Dis.* 14:2–11.

3. Greco, R., L. De Martino, G. Donnarumma, M.P. Conte, L. Seganti, and P. Valenti. 1995. Invasion of cultured human cells by *Streptococcus pyogenes*. *Res. Microbiol.* 146:551–560.

4. LaPenta, D., C. Rubens, E. Chi, and P.P. Cleary. 1994. Group A streptococci efficiently invade human respiratory epithelial cells. *Proc. Natl. Acad. Sci. USA*. 91:12115–12119.

5. Isberg, R.R. 1991. Discrimination between intracellular uptake and surface adhesion of bacterial pathogens. *Science (Wash. DC)*. 252:934–938.

6. Falkow, S. 1991. Bacterial entry into eukaryotic cells. *Cell.* 65:1099–1102.

7. Rubens, C.E., S. Smith, M. Hulse, E.Y. Chi, and G. van Belle. 1992. Respiratory epithelial cell invasion by group B streptococci. *Infect. Immun.* 60: 5157–5163.

8. Wessels, M.R., A.E. Moses, J.B. Goldberg, and T.J. DiCesare. 1991. Hyaluronic acid capsule is a virulence factor for mucoid group A streptococci. *Proc. Natl. Acad. Sci. USA*. 88:8317–8321.

9. Schmidt, K.-H., E. Günther, and H.S. Courtney. 1996. Expression of both M protein and hyaluronic acid capsule results in a high virulence for chicken embryos. *Med. Microbiol. Immunol.* 184:169–173.

10. Wessels, M.R., and M.S. Bronze. 1994. Critical role of the group A streptococcal capsule in pharyngeal colonization and infection in mice. *Proc. Natl. Acad. Sci. USA*. 91:12238–12242.

11. Wessels, M.R., J.B. Goldberg, A.E. Moses, and T.J. DiCesare. 1994. Effects on virulence of mutations in a locus essential for hyaluronic acid capsule expression in group A streptococci. *Infect. Immun.* 62:433–441.

12. Hotez, P., M. Cappello, J. Hawdon, C. Beckers, and J. Sakanari. 1994. Hyaluronidases of the gastrointestinal nematodes *Ancylostoma caninum* and *Anisakis simplex*: possible functions in the pathogenesis of human zoonoses. *J. Infect. Dis.* 170:918–926.

13. Benchetrit, L.C., S.L. Pahuja, E.D. Gray, and R.D. Edstrom. 1977. A sensitive method for the assay of hyaluronidase activity. *Anal. Biochem.* 79: 431–437.

14. Rheinwald, J.G., and M.A. Beckett. 1981. Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cultured from human squamous cell carcinomas. *Cancer Res.* 41:1657–1663.

15. Crowe, D.L., L. Hu, L.J. Gudas, and J.G. Rheinwald. 1991. Variable expression of retinoic acid receptor (RARβ) mRNA in human oral and epidermal keratinocytes; relation to keratin 19 expression and keratinization potential. *Differentiation.* 48:199–208.

16. Lindberg, K., and J.G. Rheinwald. 1990. Three distinct keratinocyte subtypes identified in human oral epithelium by their patterns of keratin expression in culture and in xenografts. *Differentiation.* 45:230–241.

17. Kruskal, B.A., K. Sastry, A.B. Warner, C.E. Mathieu, and R.A.B. Ezekowitz. 1992. Phagocyte chimeric receptors require both transmembrane and cytoplasmic domains from the mannose receptor. *J. Exp. Med.* 176:1673–1680.

18. Johnson, D.R., D.L. Stevens, and E.L. Kaplan. 1992. Epidemiologic analysis of group A streptococcal serotypes associated with severe systemic infections, rheumatic fever, or uncomplicated pharyngitis. *J. Infect. Dis.* 166:374–382.

19. Boyle, M.D.P., M.J.P. Lawman, A.P. Gee, and M. Young. 1988. Measurement of leukocyte chemotaxis *in vivo*. *Methods Enzymol.* 162:101–114.

20. Raeder, R., and M.D.P. Boyle. 1993. Association between expression of immunoglobulin G-binding proteins by group A streptococci and virulence in a mouse skin infection model. *Infect. Immun.* 61:1378–1384.

21. Dale, J.B., R.G. Washburn, M.B. Marques, and M.R. Wessels. 1996. Hyaluronate capsule and surface M protein in resistance to opsonization of group A streptococci. *Infect. Immun.* 64:1495–1501.