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Research Article

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Differentiation of Cultured Keratinocytes Promotes the Adherence of *Streptococcus pyogenes*

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Abstract

Based on a consideration of the histopathology of non-bullous impetigo that shows localization of *Streptococcus pyogenes* to highly differentiated, subcorneal keratinocytes, we hypothesized that adherence of an impetigo strain of *S. pyogenes* would be promoted by terminal differentiation of keratinocytes. An assay was developed in which *S. pyogenes* adhered via pilus-like projections from the cell wall to the surface of cultured human keratinocytes in a time- and inoculum-dependent manner suggestive of a receptor-mediated process.

Terminal differentiation of keratinocytes was induced by increasing the calcium concentration in the growth medium, and was confirmed by morphologic analysis using electron microscopy. Adherence of *S. pyogenes* was three and fourfold greater to keratinocytes differentiated in 1.0 and 1.5 mM calcium, respectively, compared with undifferentiated keratinocytes in 0.15 mM calcium. The presence of calcium during the adherence assay further enhanced adherence nearly twofold. Adherence occurred preferentially to sites of contact between adjacent keratinocytes, suggesting that the keratinocyte receptor may be a molecule involved in cell-to-cell adhesion. In contrast, nonpathogenic *Streptococcus gordonii* adhered poorly to keratinocytes regardless of their state of terminal differentiation, and adherence of a pharyngeal strain of *S. pyogenes* was twofold greater to undifferentiated than differentiated keratinocytes.

This is the first report of in vitro adherence of *S. pyogenes* to keratinocytes in a manner that emulates human impetigo. Adherence of only the impetigo strain, and not the pharyngeal strain of *S. pyogenes* or the nonpathogenic *S. gordonii* isolate, was promoted by keratinocyte differentiation. This result provides a model system for investigating the molecular pathogenesis of streptococcal skin infections. (*J. Clin. Invest.* 1998. 101:128–136.) Key words: calcium • pathogenesis • skin infection • impetigo • model

Introduction

Group A streptococci are unsurpassed among bacterial pathogens in their ability to cause a wide variety of cutaneous infections, ranging from superficial impetigo to fulminant invasive necrotizing fasciitis (1, 2). *Streptococcus pyogenes* is isolated from ~30% of all skin infections in children (3). The global incidence of severe, invasive disease due to *S. pyogenes* has increased dramatically in recent years, with the skin serving as the portal of entry in most cases (4–7). This fact has provided impetus for developing a greater understanding of the molecular pathogenesis of streptococcal skin infections.

Impetigo is the most common bacterial skin infection, accounting for ~10% of all pediatric skin problems, and 1–2% of all pediatric visits (8–11). Histopathologically, an acute inflammatory infiltrate is seen within a vesicle located between the granular and corneal layers. Initial localization of non-bullous impetigo to the subcorneal epidermis suggests that *S. pyogenes* binding and the initiation of host defense mechanisms, including generation of the chemokine gradient that attracts the inflammatory infiltrate, occur at this location where keratinocytes are most highly differentiated (12, 13). We selected impetigo as our model for streptococcal skin infections, as correlates of its well-defined histopathology can be modeled and manipulated in vitro.

Pathogenesis of skin infection involves a dynamic interaction between host and pathogen in a manner that allows for local replication of bacteria. The initial step in the pathogenesis of group A streptococcal skin infection may involve adherence of the bacteria via its adhesin(s) to a host cell receptor(s), although this is yet unproven. The type of adhesin used for specific binding appears to vary depending on the streptococcal strain and type of host cell and tissue involved (14). Our knowledge of the mechanism of adherence of *S. pyogenes* is based to date almost exclusively on studies with respiratory epithelium. These findings, however, may not be applicable to the skin. Epidermal and mucosal keratinocytes exist in markedly different environments, the former having a dry surface, while the latter is bathed continually in a complex solution (i.e., saliva or mucous). Furthermore, the array of molecules expressed by epidermal and mucosal epithelial cells, including those on their surface, differ. For example, fibronectin, which is the primary receptor for adherence of *S. pyogenes* to respiratory epithelium (15), is not expressed by keratinocytes except in wounded or inflamed epidermis (16). Oropharyngeal mucosal epithelial cells, on the other hand, lack both a corneal layer, which appears to provide sufficient protection to prevent skin infection from developing (17), and a granular layer to which infection is localized in impetigo. Furthermore, strains of *S. pyogenes* can be segregated into those that have a propensity to cause pyogenic skin and soft-tissue infections, and those that cause tonsillopharyngitis. Only a minority of strains cause infections in both sites (18, 19).

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The present studies examine adherence of *S. pyogenes* to cultured human keratinocytes in various stages of terminal differentiation. Based on a consideration of the histopathology of impetigo, we hypothesized that adherence of an impetigo strain of *S. pyogenes* would be promoted by keratinocyte differentiation.

Methods

Bacterial strains. *S. pyogenes* strain 3732, provided by Dr. Susan K. Hollingshead (University of Alabama-Birmingham), is an M-protein 52 serotype isolated from a lesion of impetigo. It is a class II strain with *emm* gene pattern D (19), characteristic of impetigo strains. *S. pyogenes* strain 87-263 is an M1 serotype isolated from the throat of a patient with uncomplicated pharyngitis (20). *Streptococcus gordonii* (Challis strain), which does not cause skin infections, was used as a negative control.

Radiolabeling of bacteria. Bacteria were stored at -70°C in suspensions of 20% glycerol (J.T. Baker, Inc., Phillipsburg, NJ) in Todd-Hewett broth (THB)¹ (Difco Laboratories Inc., Detroit, MI). Inocula from frozen cultures were placed in 10 cc THB and grown overnight at 37°C . Aliquots (0.5 cc) of the overnight stationary-phase suspensions of bacteria in THB were inoculated into 9.5 cc fresh THB and incubated at 37°C for 2 h until log-phase growth was achieved (OD at 600 nm = 0.5 to 0.6). Growth curves for each strain of bacteria showed that log-phase growth was reached under these conditions within ~ 90 –120 min (data not shown). Bacteria were washed twice by centrifugation in a Z230M Microfuge (Hermle Scientific, Gosheim, Germany) and resuspended in 1 cc RPMI 1640 (Mediatech Inc., Herndon, VA) without L-leucine. Radiolabeled L-[^3H]leucine (50 μCi , 131 Ci/mmol, 1 mCi/cc; Amersham Corp., Arlington Heights, IL) was added, and the bacteria were incubated at 37°C for 1 h. THB (9 cc) was added, and the suspensions were incubated for 1 h at 37°C to bring the bacteria to log-phase growth. After pelleting the bacteria, they were suspended in 10 cc THB with 20% glycerol and frozen at -70°C in 1-cc aliquots. Bacterial viability and adherence were unaffected by freezing.

Frozen L-[^3H]leucine-labeled bacteria were thawed, and 0.5 cc THB was added to 1 cc of the bacterial suspensions in a microfuge tube. The bacteria were pelleted, resuspended in 1 cc THB, and washed once. Washed bacteria were resuspended in 1 cc THB and incubated at 37°C for 15 min. Bacteria were pelleted and resuspended in 10 cc cold HBSS with 5% BSA (endotoxin < 0.1 ng/ml, cell culture-tested; Sigma Chemical Co., St. Louis, MO) or 0.25% gelatin (Difco Laboratories, Inc.) to block nonspecific adherence to the tissue culture plate. Bacterial suspensions were incubated at 4°C for 30 min before initiation of adherence assays. 50- μl aliquots of the suspensions were counted for radioactivity (cpm; three replications). Suspensions were diluted to 10^{-4} , and 20- μl aliquots were plated on Todd-Hewett agar (THA) for determination of CFU/cc (three replications). Ratio of CFU-to-cpm was determined for each preparation, and ranged from 200 to 300.

Keratinocyte culture. Keratinocytes were isolated enzymatically from neonatal foreskins in complete keratinocyte growth media (cKGM, keratinocyte basal media supplemented with 0.1 ng/ml human recombinant epidermal growth factor, 5 $\mu\text{g/ml}$ insulin, 0.5 $\mu\text{g/ml}$ hydrocortisone, 50 $\mu\text{g/ml}$ gentamicin, 50 ng/ml amphotericin B, and 0.15 mM calcium; Clonetics, San Diego, CA) as described previously (21). The 10^6 cells were seeded in 5 cc cKGM with 30 $\mu\text{g/ml}$ bovine pituitary extract (BPE) into a 25-cm² flask (Becton-Dickinson, Inc., Rutherford, NJ) in a humidified atmosphere containing 5% CO_2 at

36.7°C . Cells were washed with HBSS the next day, and fed fresh cKGM without BPE every 2–3 d. After ~ 5 –7 d, the primary keratinocytes at $\sim 80\%$ of confluence were trypsinized and frozen in freezing solution (80% cKGM, 10% FCS, 10% DMSO; Sigma Chemical Co.) at a concentration of $\sim 10^6$ keratinocytes/cc.

For use in experiments, keratinocytes were thawed at room temperature and seeded in cKGM with BPE into a 25-cm² flask. Fresh cKGM without BPE was provided the next day and every 2–3 d thereafter. At 80% of confluence, cells were passaged into 6-well tissue culture plates (Corning Glass Works, Corning, NY) for adherence experiments. 60,000 keratinocytes were seeded in cKGM with BPE into each well. Growth medium was changed the next day to cKGM without BPE. At confluence, keratinocyte monolayers grown in 0.15 mM calcium in 6-well tissue culture plates contained $\sim 2 \times 10^6$ keratinocytes per well.

Keratinocyte differentiation. Differentiation of cultured keratinocytes can be regulated by adjusting the extracellular calcium concentration (22–29). When keratinocytes in 6-well tissue culture plate wells reached 80% of confluence, the extracellular calcium concentration was maintained at 0.15 mM, or increased to 0.6–1.5 mM to induce keratinocyte differentiation. Keratinocytes were grown for an additional 7 d in the adjusted calcium concentration before adherence or morphologic assays. Differentiation was assessed morphologically by electron microscopy.

Calcium determination. Calcium concentration in keratinocyte growth and incubation media was confirmed using an ion-selective electrode on an AVL 988-4 Electrolyte Analyzer (AVL Scientific Corp., Rosewell, GA), as described previously (30). The range of detection of ionized calcium was 0.12–6.0 mM, with resolution to 0.001 mM. Neither 0.25% gelatin or 5% BSA used for blocking nonspecific adherence, or BPE used during plating of keratinocytes, contributed measurable concentrations of calcium to the media (data not shown).

Transmission electron microscopy. Keratinocytes were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (Ted Pella Inc., Redding, CA) for 2 h at room temperature, washed with 0.1 M cacodylate buffer (pH 7.4), and postfixed in 1% OsO_4 (Ted Pella Inc.) in double-distilled H_2O as described previously (31). Samples were dehydrated using a graded alcohol series, cells were embedded in Medcast (Ted Pella Inc.), thin sections were cut at a ultramicrotome with a diamond knife (LKB-Produkter AB, Bromma, Sweden), stained with uranyl acetate (Ted Pella Inc.) and lead citrate (Ted Pella Inc.), and examined with a JEOL-1200EX transmission electron microscope (JEOL Ltd., Tokyo, Japan) at 80 kV.

Bacterial adherence assay. Wells without keratinocytes served as a negative control. Each well with or without cultured keratinocytes was washed three times with 1 cc cold HBSS. Before inoculating cultures with bacteria, nonspecific sites for adherence on keratinocytes were blocked by exposure to 0.25% gelatin or 5% BSA in HBSS for 2 h at 4°C . After blocking, the HBSS/BSA solution was removed by aspirating to dryness, and 1.5 cc of a suspension of radiolabeled bacteria containing $\sim 5 \times 10^7$ – 1×10^8 CFU/cc HBSS with 0.25% gelatin or 5% BSA was added to each well. Multiplicity of infection ranged from ~ 50 –100 bacteria per keratinocyte. Unless indicated otherwise, adherence occurred for 4 h at 4°C with no centrifugation of bacteria against the keratinocytes. Aliquots of the suspension were plated on THA to determine CFU/cc (three replications). To terminate adherence and remove nonspecifically adherent bacteria, each well was washed three times with 1 cc HBSS at 4°C . For each wash, the plate was vortexed for 10 s to dislodge nonspecifically adherent bacteria. Nonadherent bacteria were removed within three washes; additional washes did not alter the number of adherent bacteria (data not shown). NaOH (2 N, 1 cc) was added to each well, and the plate was incubated at 65°C for 1 h. Judged by inspection under light microscopy, this procedure entirely lysed the keratinocytes. 50- μl aliquots were removed for counting (three replications) on solid scintillant (Readycaps; Beckman Instruments Inc., Fullerton, CA). Counts per min in wells without keratinocytes (background) was subtracted from cpm in wells containing keratinocytes (bound) to correct for nonspe-

1. Abbreviations used in this paper: BPE, bovine pituitary extract; cKGM, complete keratinocyte growth media; THA, Todd-Hewett agar; THB, Todd-Hewett broth.

cific adherence. Percent adherence was calculated as follows: $(\text{cpm bound} - \text{background}) / (\text{cpm added} - \text{cpm ambient}) \times 100$.

Bacterial internalization assay. To confirm that the bacterial adherence assay measured adherent, not internalized bacteria, the ability of *S. pyogenes* strain 3732 to enter cultured keratinocytes at 4°C was determined by a modification of methods described previously by Rubens et al. (32). In brief, a standard adherence assay as described above was performed at 4°C using an inoculum of 1×10^7 CFU/cc ^3H -radiolabeled *S. pyogenes*, followed by the addition of 5 µg/ml penicillin and 100 µg/ml gentamicin in HBSS at 37°C for 4 h to kill extracellular bacteria remaining after adherence had been terminated by washing with HBSS. These antibiotics penetrate host cells to an insignificant degree (33), allowing for the survival of intracellular bacteria. After removing antibiotics from tissue culture plate wells with four washes of 4 cc HBSS at room temperature, keratinocytes were dislodged from the tissue culture plates by incubation with 0.25% trypsin at 37°C for 5 min. To release intracellular bacteria from keratinocytes, Triton X-100 (Sigma Chemical Co.) was added to a final concentration of 0.025%. The number of viable intracellular bacteria was determined by plating an aliquot of the final suspension on THA. CFU recovered from wells without keratinocytes were subtracted from the CFU from wells containing keratinocytes. Total cell-associated bacteria was determined by scintillation counting of an aliquot of the final suspension. The percentage of the total inoculum added to each well that adhered to the surface of keratinocytes was determined by subtracting the number of intracellular bacteria from the cell-associated bacteria.

Results

Adherence to keratinocyte monolayers. An assay was developed that measured specific adherence of the *S. pyogenes* impetigo strain 3732 to cultured human foreskin keratinocytes.

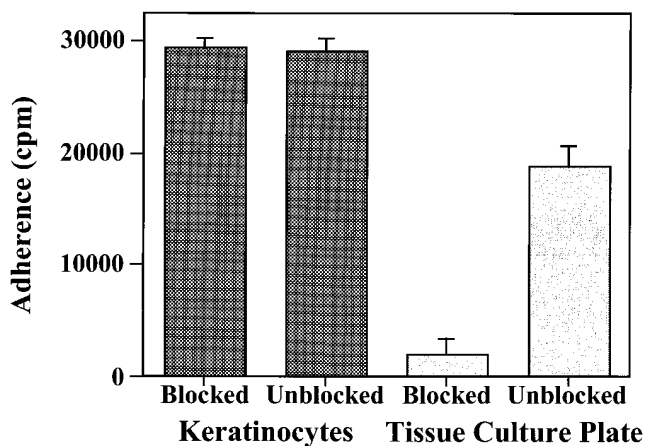


Figure 1. BSA blocked nonspecific adherence of *Streptococcus pyogenes* to the tissue culture plate, but did not affect adherence to keratinocytes. An M52 *S. pyogenes* impetigo strain (3732) was radiolabeled with L-[^3H]leucine and suspended in HBSS. The bacteria and 6-well tissue culture plate wells with or without monolayers of confluent cultures of undifferentiated human foreskin keratinocytes grown in 0.15 mM calcium were preincubated with 5% BSA in HBSS for 2 h at 4°C. Adherence was then initiated by adding 2.1×10^8 CFU of bacteria to each well, followed by centrifugation at 800 relative centrifugal force for 10 min. Adherence occurred for 4 h at 4°C, then non-adherent bacteria were removed by washing. Keratinocytes were solubilized with NaOH, and radioactivity in an aliquot was determined by scintillation counting. Adherence to keratinocytes (hatched bars) and to the tissue culture plate (shaded bars) is expressed as the total cpm per well. Error bars represent \pm SD.

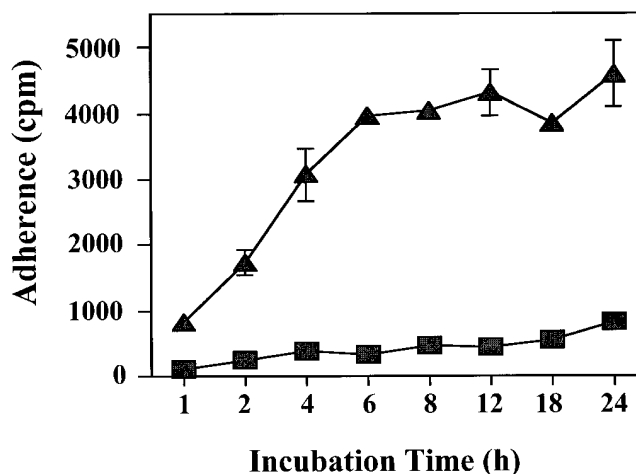


Figure 2. Adherence of *Streptococcus pyogenes* (strain 3732) to keratinocytes, not to the tissue culture plate, was saturated by 4–6 h incubation at 4°C. Nonspecific sites for adherence on monolayers of undifferentiated keratinocytes grown in 0.15 mM calcium and on bacteria were blocked by incubation with 5% BSA for 2 h at 4°C. Adherence to keratinocytes (Δ) or to the tissue culture plate (\square) was initiated by adding 1.3×10^8 CFU of radiolabeled *S. pyogenes* to confluent keratinocyte cultures at 4°C. Centrifugation was not used to initiate adherence. Error bars represent \pm SD.

We preferred to use keratinocytes in early passage rather than an immortalized keratinocyte cell line to preserve expression of cell surface receptors for bacterial adherence that resemble those of keratinocytes in vivo.

We first sought to demonstrate that *S. pyogenes* adhered to monolayers of keratinocytes, and that nonspecific binding to exposed tissue culture well plastic could be blocked. Preincubation of keratinocytes and bacteria separately with 5% BSA for 2 h before initiating adherence blocked nonspecific binding of bacteria to the tissue culture plate (Fig. 1). Blocking with BSA, however, had no effect on adherence of *S. pyogenes* to keratinocytes (Fig. 1). Similar adherence results were obtained when keratinocytes were preincubated with 0.25% gelatin as the blocking agent for 2 or 4 h (data not shown). Thus, either agent was suitable for blocking. Blockade of nonspecific adherence was effective at 4°C, as adherence to the tissue culture plate was insignificant in comparison to adherence to keratinocytes during a 24-h incubation (Fig. 2). At 37°C, however, excessive nonspecific adherence to the tissue culture plate occurred despite preincubation with 5% BSA (data not shown). Consequently, adherence assays were routinely performed at 4°C since adherence at 37°C was confounded by the high nonspecific background binding.

The adherence assay cannot distinguish between adherent or internalized bacteria, as it measures total cell-associated radiolabeled bacteria. The contribution of intracellular bacteria to total cell-associated bacteria was determined by measuring the number of bacteria that were internalized under the conditions of the adherence assay. After incubation at 4°C for 4 h, $< 0.01\%$ of an inoculum of 8×10^7 CFU was recovered in an internalization assay from keratinocytes cultured in either 0.15 mM or 1.0 mM calcium (data not shown). This result indicates that the cpm measured in the adherence assays reflected bound, not intracellular bacteria. Lack of recovery of a signifi-

cant number of intracellular bacteria was not due to bacterial death during their isolation in the internalization assay, as use of 0.25% trypsin or 0.025% Triton X-100 in the internalization assay did not affect viability of *S. pyogenes* (data not shown). Viability of keratinocytes also was unaffected during the assay as judged by their ability to exclude trypan blue stain, and by their maintenance of normal morphology under light and electron microscopy (data not shown).

Adherence that occurs via a receptor-mediated process should be saturable with increasing time of incubation and increasing bacterial inoculum. Adherence of *S. pyogenes* to monolayers of undifferentiated cultured human foreskin keratinocytes increased over time, becoming saturated at 4–6 h incubation with an inoculum of 9×10^7 CFU/cc (1.3×10^8 CFU/well; Fig. 2). The plateau in adherence was not due to loss of bacterial viability, as there was no difference in total CFU/cc recovered from the supernatant in wells after 0–24 h incubation (data not shown). Keratinocyte viability also was preserved over the 24-h incubation, as judged functionally by exclusion of trypan blue stain and morphologically by light and electron microscopy (data not shown).

At 4 h incubation, ~ 1 CFU of *S. pyogenes* bound per keratinocyte after inoculation with 10^7 CFU/cc; this figure rose 10-fold to ~ 10 CFU/keratinocyte when the inoculum was increased to 10^8 CFU/cc (Fig. 3). Total cell-associated CFU/keratinocyte increased at an accelerated rate as the inoculum was increased above 1×10^9 CFU/cc. Under light microscopy, many of the cell-associated bacteria in wells inoculated with more than 1×10^9 CFU/cc appeared to be agglutinated to each other on the keratinocyte surface rather than bound directly to keratinocytes. Since the relationship between bound CFU/ke-

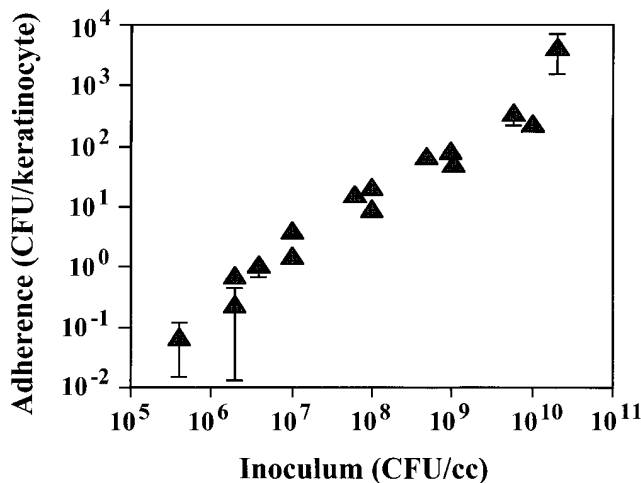


Figure 3. Adherence of *Streptococcus pyogenes* (strain 3732) to keratinocytes increased linearly as the bacterial inoculum was increased. *S. pyogenes* was added to confluent cultures of undifferentiated keratinocytes in 0.15 mM calcium at 4°C. Bacterial inoculum was varied as indicated. Adherence was terminated after 4 h by washing and vortexing off nonadherent bacteria. An aliquot of each bacterial inoculum was plated on Todd-Hewett agar for colony counting, and cpm of radioactivity was related to CFU. Average number of keratinocytes per well was determined by dislodging keratinocytes with 0.25% trypsin and counting them with a hemocytometer. Data are expressed as CFU of *S. pyogenes* per keratinocyte as a function of the inoculum. Error bars represent \pm SD.

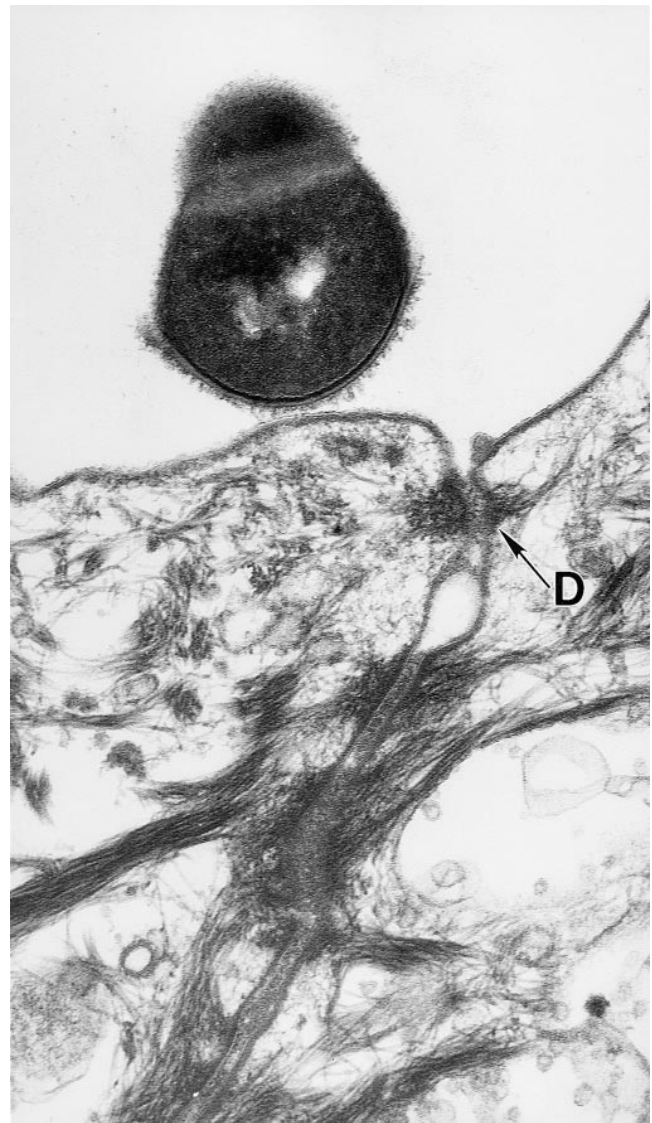


Figure 4. *Streptococcus pyogenes* (strain 3732) adhered by pilus-like cell wall projections to a cultured human foreskin keratinocyte adjacent to a site of contact (D, desmosome) with another keratinocyte (25,000 \times). Keratinocytes were grown to 80% of confluence in keratinocyte growth media containing 0.15 mM extracellular calcium, then induced to differentiate in 1.0 mM calcium for an additional 7 d before adding *S. pyogenes*. Electron microscopy was performed after incubation of bacteria with keratinocytes for 24 h at 4°C.

ratinoocyte and inoculum was linear with inocula ranging from 10^6 to 10^8 CFU/cc (Fig. 3), adherence assays were routinely performed using inocula of 1×10^7 – 1×10^8 CFU/cc.

Together, these data demonstrate time-dependent saturable adherence of *S. pyogenes* to undifferentiated human keratinocytes, suggestive of a receptor-mediated process. By electron microscopy, bacteria were found to adhere to keratinocytes by pilus-like cell-wall projections (Fig. 4), suggesting that the bacterial adhesin for attachment resides on these structures.

Adherence to differentiated keratinocytes. Based on a consideration of the histopathology of impetigo, in which bacteria

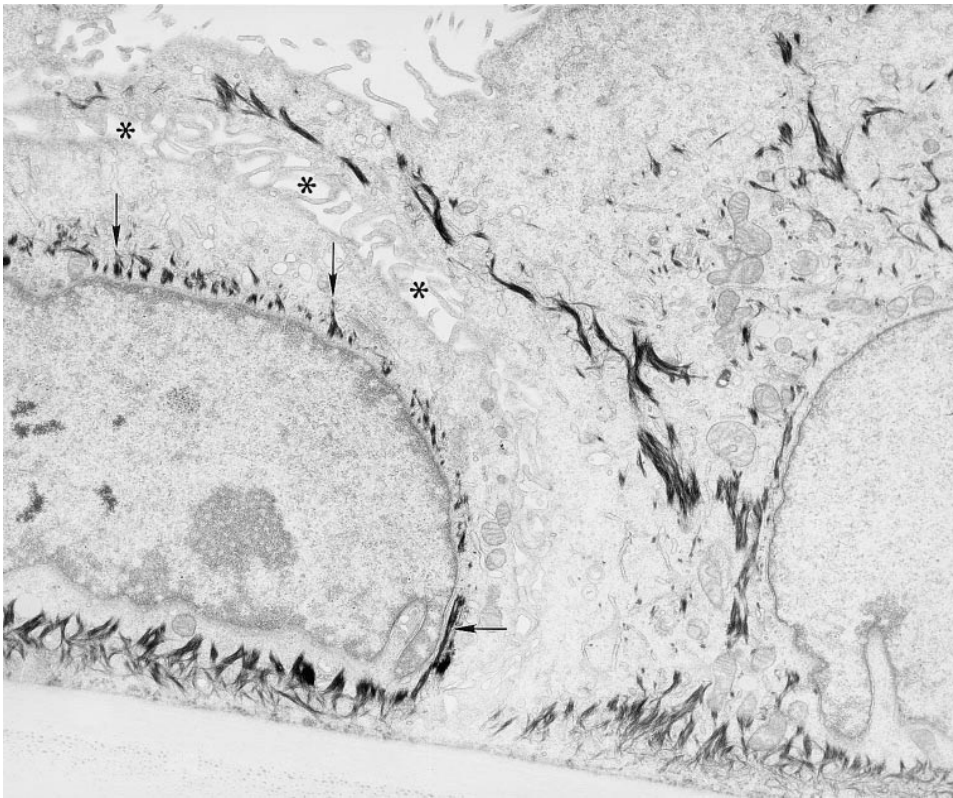


Figure 5. Electron micrograph of undifferentiated keratinocytes grown to confluence in media containing 0.15 mM calcium. Keratinocytes grew in a monolayer and had a perinuclear distribution of keratin intermediate filaments (*arrows*). Spaces between adjacent keratinocytes (*) were readily apparent, but desmosome formation was sparse (5,000 \times).

are localized to the most highly differentiated keratinocytes of the subcorneal layer, we hypothesized that adherence would be promoted by terminal differentiation of keratinocytes. The principal factors that impact keratinocyte differentiation in our keratinocyte culture system are degree of keratinocyte confluence and concentration of calcium in the growth media (22–

29). Confluence was controlled by seeding keratinocytes at a constant density when initiating cultures, and by adjusting the calcium concentration when cells had reached 80% of confluence. Cultures were confluent by the time adherence assays were performed 7 d after adjusting the calcium concentration. State of terminal keratinocyte differentiation was determined

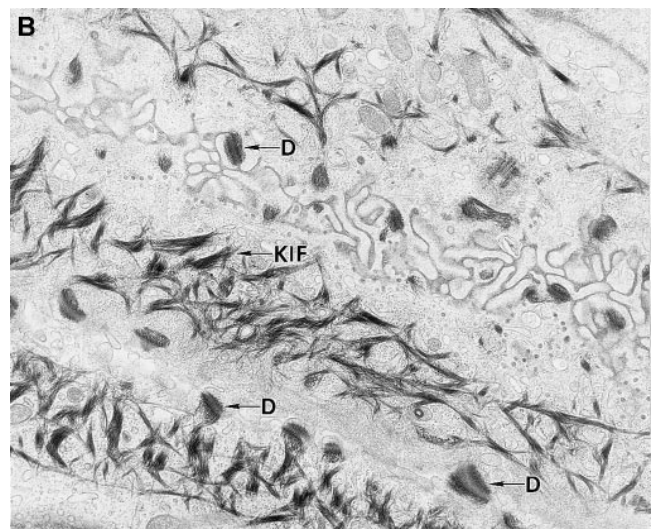
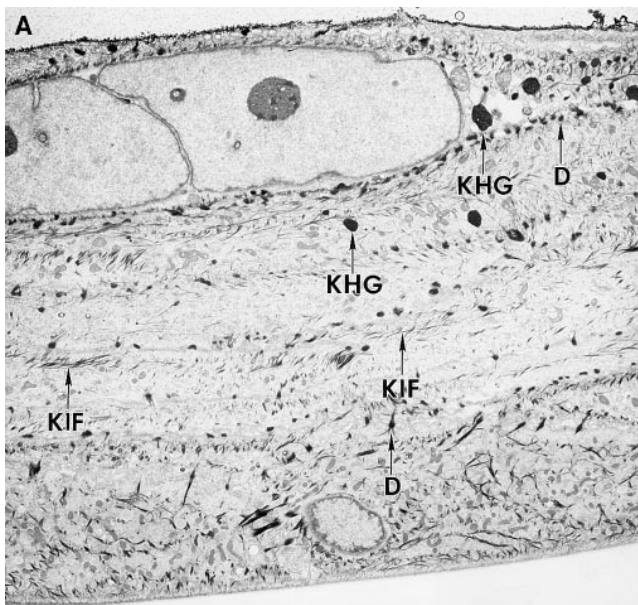


Figure 6. Electron micrographs of differentiated keratinocytes grown to confluence in medium containing 1.0 mM calcium. (A) Keratinocytes were stratified to at least three cell layers, keratin intermediate filaments (*KIF*) were dispersed throughout the cytoplasm, and keratohyalin granules (*KHG*) were abundant (2,000 \times). (B) Numerous desmosomes (*D*) were present between adjacent cells (10,000 \times).

morphologically by electron microscopy. Cells in 0.15 mM calcium grew as a monolayer (Fig. 5); overlap on the apical surface occurred at occasional sites. Spaces between adjacent cells were relatively large, keratin intermediate filaments were located in a perinuclear distribution, and few desmosomes were present (Fig. 5). Desmosomes that formed were structurally normal, and keratohyaline granules were present. In contrast, keratinocytes differentiated in 1.0 mM calcium were stratified to 3–6 cell layers (Fig. 6 A). Keratin intermediate filaments were dispersed throughout the cytoplasm, and extended to the plasma membrane where numerous desmosomal connections joined closely adherent, adjacent cells (Fig. 6 B).

The gross difference in morphology between keratinocytes differentiated in 1.0 mM calcium, and those that remained undifferentiated in 0.15 mM calcium, particularly in the interaction of adjacent keratinocytes with one another, suggests that these two keratinocyte populations differed markedly in their expression of cell-surface proteins, presumably including cell adhesion molecules (33). We hypothesized that this difference would be reflected in the ability of *S. pyogenes* to adhere.

Keratinocytes that had been differentiated in 1.0 mM calcium, or maintained in an undifferentiated state in 0.15 mM calcium, were compared in adherence assays. The presence of calcium has been shown to affect adherence of some bacterial species (e.g., *Yersinia pseudotuberculosis*) to mammalian host cell receptors (34). Consequently, calcium was not included in the incubation media immediately before adherence assays to eliminate its presence as a confounding factor in assessment of the effect of keratinocyte differentiation on adherence. Adherence was three and fourfold greater to differentiated keratinocytes grown in 1.0 mM and 1.5 mM calcium, respectively,

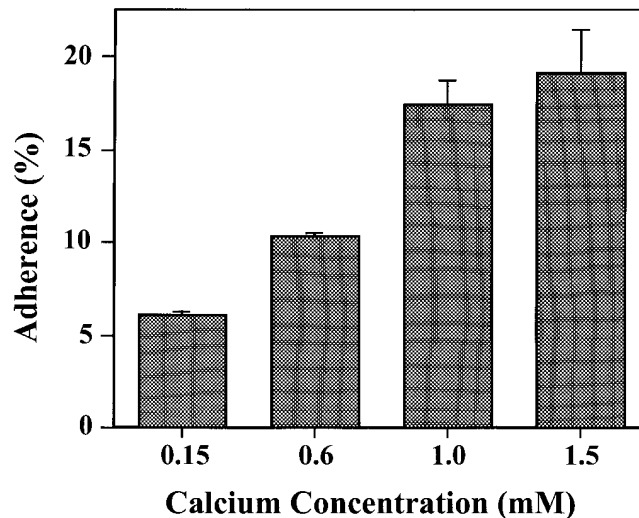


Figure 7. Adherence of *Streptococcus pyogenes* (strain 3732) to keratinocytes increased with keratinocyte terminal differentiation. Cultured keratinocytes were grown to 80% of confluence in keratinocyte growth medium containing 0.15 mM extracellular calcium. The calcium concentration was either maintained at 0.15 mM or increased to 0.6–1.5 mM for an additional 7 d before initiating adherence by adding 1.4×10^8 CFU of radiolabeled *S. pyogenes* per well at 4°C. Adherence is expressed as the percent of the total cpm of bacteria added to each well that remained after washing and vortexing away nonadherent bacteria. Error bars represent \pm SD.

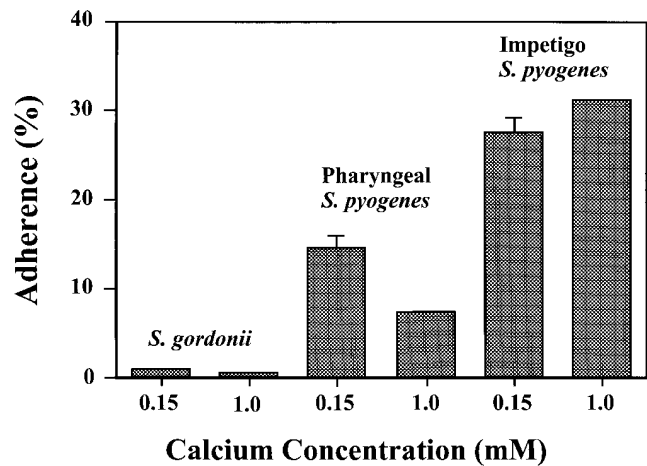


Figure 8. Keratinocyte adherence of *Streptococcus pyogenes* impetigo strain 3732 exceeded that of *S. pyogenes* pharyngeal strain 87–263 and *Streptococcus gordonii* (Challis). Cultured keratinocytes were grown to 80% of confluence in keratinocyte growth medium containing 0.15 mM extracellular calcium. The calcium concentration was either maintained at 0.15 mM or increased to 1.0 mM for an additional 5 d before initiating adherence at 4°C by adding 3.5×10^7 , 6.7×10^7 , or 1.8×10^8 CFU/well of radiolabeled *S. pyogenes* impetigo strain 3732, *S. pyogenes* pharyngeal strain 87–263, or *S. gordonii* (Challis), respectively. Adherence is expressed as the percent of the total cpm of bacteria added to each well that remained after washing and vortexing away nonadherent bacteria. Error bars represent \pm SD.

compared with undifferentiated keratinocytes grown in 0.15 mM calcium (Fig. 7). Similarly, adherence to keratinocytes grown in 0.05 mM or 0.09 mM calcium to produce more undifferentiated cells was reduced to 65% and 61%, respectively, compared with adherence to keratinocytes in 0.15 mM calcium (data not shown). Greater adherence to differentiated keratinocytes was not due to exposure of a larger cell surface area for adherence. On the contrary, the surface area exposed on differentiated keratinocytes was less than that of undifferentiated keratinocytes because the latter had relatively large intercellular spaces to which the bacteria had access (Fig. 5).

In contrast to impetigo strain 3732, the nonpathogenic streptococcus, *S. gordonii*, adhered poorly to both differentiated and undifferentiated keratinocytes; < 1% of the inocula adhered to either keratinocyte population (Fig. 8). Furthermore, a serotype M1 strain of *S. pyogenes* (87–263) isolated from the throat of a patient with uncomplicated pharyngitis (20) bound less than strain 3732 to either keratinocyte population, and in contrast, showed greater (twofold) adherence to undifferentiated than differentiated keratinocytes (Fig. 8). This result is in agreement with Okada et al. (34), who reported that adherence of an M6 strain of *S. pyogenes* isolated from a patient with tonsillopharyngitis (35) was approximately two to threefold greater to undifferentiated (0.15 mM calcium) than differentiated (1.2 mM calcium) keratinocytes. In multiple experiments, keratinocyte differentiation consistently promoted the adherence of strain 3732 and decreased the adherence of strain 87–263, although the magnitude of the effects varied depending on the person from whom the keratinocytes were isolated.

Adherence of *S. pyogenes* to either differentiated or undif-

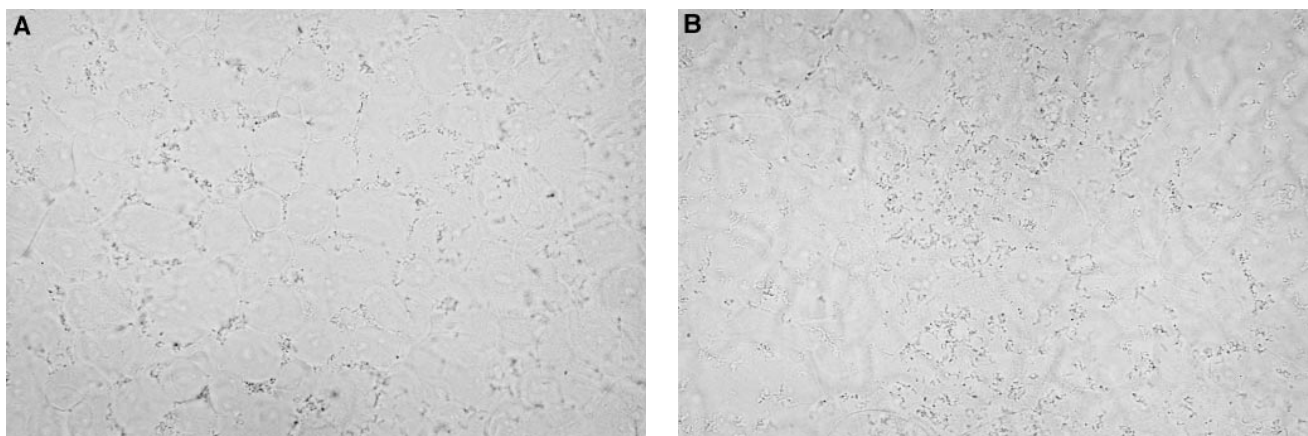


Figure 9. *Streptococcus pyogenes* (strain 3732) adhered to cultured human keratinocytes preferentially at sites of cell–cell contact. Keratinocytes were grown to 80% of confluence in keratinocyte growth medium containing 0.15 mM extracellular calcium. The calcium concentration was either (A) maintained at 0.15 mM or (B) increased to 1.0 mM for an additional 7 d before adding *S. pyogenes*. Light microscopy was performed after incubation of bacteria with keratinocytes for 24 h at 4°C (40×).

ferentiated keratinocytes was found to occur preferentially to sites of contact between keratinocytes (Figs. 4 and 9). Distribution of the bacteria attached to differentiated keratinocytes appeared to be more random, however, with an increased proportion of adherent bacteria scattered over the apical surface (Fig. 9 B). We postulated that adherence of *S. pyogenes* at sites of cell–cell contact might be reflective of binding to cellular adhesion molecules (e.g., cadherins, integrins), and that adherence might be subject to modulation by calcium as shown for *Y. pseudotuberculosis* (36).

To assess the importance of calcium in modulating adherence of *S. pyogenes* to keratinocytes, adherence to differentiated and undifferentiated keratinocytes was examined with and without calcium present in the media during blockade of nonspecific sites for adherence (2 h) and during the adherence assay (4 h). Adherence to either differentiated or undifferentiated keratinocytes was enhanced 1.7-fold by the presence of calcium (Fig. 10), suggesting that calcium modulated the interaction between bacteria and keratinocytes. Again, greater adherence to differentiated than undifferentiated keratinocytes, independent of the presence of calcium, suggests that keratinocyte differentiation promoted the adherence of *S. pyogenes* (Fig. 10).

Discussion

We hypothesized that skin infection with *S. pyogenes* occurs when the corneal layer is disrupted and bacteria gain access to epidermal keratinocytes. There, they adhere preferentially to receptors present predominantly on terminally differentiated keratinocytes. In this paper, we report the first *in vitro* investigation that demonstrates the adherence of *S. pyogenes* to skin cells in a manner that emulates human impetigo, whereby bacteria adhere preferentially to more highly differentiated keratinocytes.

Our adherence assay, using an M52 impetigo isolate of *S. pyogenes*, quantified the number of bacteria bound specifically to keratinocytes. Adherence occurred in a time- and inoculum-dependent manner, suggestive of a specific receptor-mediated

process. Preferential adherence to more highly differentiated keratinocytes was specific to the pathogenic impetigo strain of *S. pyogenes*. The relative inability of *S. gordonii* to adhere to keratinocytes may explain, at least in part, its lack of pathogenicity in the skin, and further supports the hypothesis that adherence is an important initiating step in the pathogenesis of cutaneous infections. Decreased adherence of the pharyngeal strain of *S. pyogenes* to differentiated compared with undifferentiated keratinocytes further highlights the specific nature of

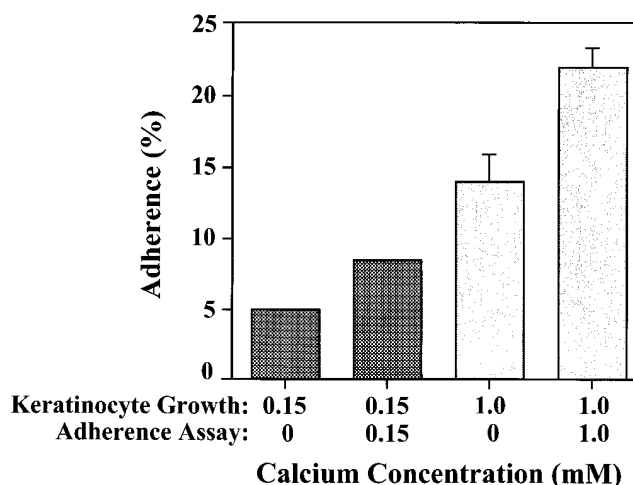


Figure 10. Adherence of *Streptococcus pyogenes* (strain 3732) to keratinocytes was increased in the presence of calcium. Cultured keratinocytes were grown to 80% of confluence in keratinocyte growth medium containing 0.15 mM extracellular calcium. The calcium concentration was either maintained at 0.15 mM (hatched bars) or increased to 1.0 mM (shaded bars) for an additional 7 d before measuring adherence of *S. pyogenes*. Calcium was either excluded from incubation media or was included during blockade of nonspecific adherence as well as during the adherence assay. Adherence was initiated by adding 2.0×10^8 CFU of radiolabeled *S. pyogenes* per well at 4°C. Error bars represent \pm SD.

the enhanced interaction of skin-trophic *S. pyogenes* with receptors on differentiated keratinocytes. Okada et al. (34) also found that binding of a pharyngeal M6 strain of *S. pyogenes* to differentiated keratinocytes in 1.2 mM calcium was two to threefold lower compared with undifferentiated keratinocytes in 0.15 mM calcium. They used semiquantitative methods, basing their conclusions on visualization of adherent bacteria by light microscopy, and did not demonstrate the state of differentiation of their keratinocytes. Nevertheless, our quantitative assay corroborates their finding, and demonstrates that there is a difference in the nature of the interaction of impetigo compared with pharyngeal strains of *S. pyogenes* with keratinocytes. Enhanced adherence of the impetigo strain 3732 (but not pharyngeal strains of *S. pyogenes* or a nonpathogenic streptococcus) to more highly differentiated keratinocytes emulates impetigo, and provides a model system for investigating the pathogenesis of cutaneous infections, particularly the molecular mechanisms of interaction of skin-trophic *S. pyogenes* with keratinocytes.

It has long been known that strains of *S. pyogenes* isolated from skin infections differ from pharyngeal isolates in their M-protein serotype and expression of opacity factor (18). More recently, differences in their genomes have been described, including distinct patterns of chromosomal arrangements of the *emm* genes that encode the M- and M-like proteins (19). Furthermore, unlike most pharyngeal isolates, impetigo strains typically are able to bind IgG and IgA due to the expression of additional genetic elements (*fcr* and *enn* genes, respectively) within the *virR* regulon, the largest cluster of linked and coordinately regulated genes described to date in *S. pyogenes* (37–39). Consequently, it is not surprising that adherence of an impetigo isolate of *S. pyogenes* to keratinocytes would differ from that of a respiratory isolate, and that the pattern of adherence, with increased adherence to more highly differentiated keratinocytes, would more closely model the histopathology of superficial streptococcal skin infections in humans.

In studies of cutaneous binding of a genetically engineered M6 pharyngeal strain of *S. pyogenes*, the C-repeat domain of M protein mediated adherence to the CD46 molecule on keratinocytes, while protein F was responsible for binding to Langerhans cells of the skin (40, 41). The relevance of these molecular interactions to the pathogenesis of skin infections is not clear, and must be confirmed with a skin-trophic, impetigo isolate of *S. pyogenes*.

We observed by electron microscopy that *S. pyogenes* adhered by the tips of pilus-like projections from the cell wall, supporting the hypothesis that attachment involves a specific bacteria–host cell interaction. Given that calcium-stimulated keratinocyte terminal differentiation promoted adherence, we postulate that expression of host–cell receptors for adherence of strains of *S. pyogenes* that are pathogenic for the skin may increase with terminal differentiation.

A plethora of metabolic and biochemical changes accompany terminal differentiation of keratinocytes, including formation of desmosomes (25), synthesis and posttranslational processing of cornified envelope proteins and keratin intermediate filament aggregating protein (27, 28), production of lipids and their packaging in lamellar bodies for export to the intercellular space where they are integral to epithelial barrier function (42), and alterations in synthesis and distribution of cellular adhesion molecules (43). The changes appear to be reflected in the marked difference in cell-to-cell association

observed by electron microscopy between keratinocytes in our two populations. Undifferentiated keratinocytes (in 0.15 mM calcium) grew as a monolayer, had gaps on the lateral surface between adjacent keratinocytes, and formed few desmosomes, while differentiated keratinocytes (in 1.0 mM calcium) became stratified and were closely adherent with numerous desmosomal connections. Based on the localization of adherent bacteria primarily to sites of contact between adjacent keratinocytes, it seems plausible that the host–cell receptor for adherence may be a molecule that mediates adhesion between keratinocytes. The bacterial pathogens identified thus far that interact with mammalian cellular adhesion molecules (e.g., integrins), however, typically have been intracellular parasites that, subsequent to attachment, are internalized through rearrangement of underlying host cytoskeletal elements attached to the cellular adhesion molecules (e.g., actin microfilaments; 42, 44). Internalization appears to be an important step in intercellular spread of these pathogens. It is not known whether internalization of *S. pyogenes* plays a role in the pathogenesis of skin infections (45).

In addition to promoting adherence through stimulation of keratinocyte differentiation, calcium further modulated the adherence process when it was present in the incubation media during adherence assays. Calcium may have mediated this effect through several potential mechanisms, including the following: (a) an increase in number of a single type of receptor; (b) induction of additional type(s) of host cell receptor(s); (c) facilitation of a more avid association between bacterial adhesin and host cell receptor; (d) increased exposure of receptors through changes in their conformation; or (e) a change in the number, exposure, and/or function of bacterial adhesins. The nature of the host cell receptor and bacterial adhesin, and the role of calcium in the adherence process, are the subject of ongoing investigations in our laboratory. Modulation of adherence by calcium has been described for *Y. pseudotuberculosis* (36). This effect occurs because of the presence of calcium-binding domains on β 1-integrin cellular adhesion molecules, the mammalian host cell receptor for the bacterial adhesin (36, 46). Enhancement of adherence by calcium also has been reported for *Candida albicans* (47), which binds to host cells by an integrin-like adhesin (48).

Use of differentiated, cultured human keratinocytes in an experimental model that emulates human impetigo may provide insight into the bacterial adhesin(s) and keratinocyte receptor(s) involved in adherence, and the role of adherence in pathogenesis of cutaneous streptococcal infections. Through understanding of the molecular pathogenesis of these infections, it may be possible to devise new rational approaches to their prevention and management.

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