I-domain-containing integrins serve as pilus receptors for *Neisseria gonorrhoeae* adherence to human epithelial cells

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**Summary**

Two pilus receptors are identified for the pathogenic *Neisseria*, CD46 and complement receptor 3. An intimate association between the asialoglycoprotein receptor and gonococcal lipooligosaccharide mediates invasion of primary, male urethral epithelial cells (UECs); however, studies to identify pilus receptors on these cells have not been performed. Based on our previous studies we reasoned that the I-domain-containing (IDC), α₅- and α₂-integrins might serve as pilus receptors on UECs and on urethral tissue. Confocal microscopy revealed colocalization of pilus with α₁ and α₂ integrins on UECs and tissue. We found that recombinant I-domain and antibodies directed against the α₂- and α₅-integrins inhibited gonococcal association with UECs and with immortal cell lines of variable origin. Gonococcus-integrin colocalization occurred at early time points post infection, but this interaction dissociated with extended infection. Similarly, Western Blot analyses revealed that gonococcal pilin comimmunoprecipitates with α₂- and α₅-integrins. However, studies performed in parallel and that were designed to capture CD46-pilus immune complexes indicated that a CD46–pilus interaction did not occur. Collectively, these data suggest that while CD46 might be able to bind gonococcal pilus, IDC integrins are preferentially used as the initial docking site for gonococci on UECs, on urethral tissue and on some immortal cell lines.

**Introduction**

*Neisseria gonorrhoeae*, the gonococcus, is an exclusive human pathogen that causes the sexually transmitted disease gonorrhoea. Mechanisms used by the gonococcus to potentiate disease are variable and are, in part, dependent upon the infection site (reviewed in Jerse and Rest, 1997; Dehio *et al.*, 2000; Merz and So, 2000; Edwards and Apicella, 2004; Edwards *et al.*, 2004). Adherence of gonococci to host cells occurs independently of their invasion of those same cells. It is generally accepted that the ability of pilus to initially bind to host cell surface constituents places the bacterium in proximity to the cell, overcoming repulsive forces that would impede an intimate, subsequent, host-bacterial interaction (Ward and Watt, 1977; Wicken, 1985). Thus, the pilus–host receptor interaction is required for and occurs early in cellular colonization. Previous studies using the immortal cervical epithelial cell line, ME180, indicate that CD46 serves as the pilus receptor in these cells (Källström *et al.*, 1997). However, immortal cell lines (including ME180 cells) do not express complement receptor 3 (CR3, CD11b/CD18), which serves as an important mechanism by which *N. gonorrhoeae* elicit membrane ruffling and cellular invasion of primary, human, cervical epithelial cells (Edwards *et al.*, 2001). In these primary cells and in cervical tissue, CD46 is expressed on the basolateral surface (Hasty *et al.*, 1994; Simpson *et al.*, 1997; Oglesby, 1998; J. L. Edwards and M. A. Apicella, unpublished) and is not transcytosed to the apical cervical surface (Maisner *et al.*, 1996; Teuchert *et al.*, 1999). Similar observations are noted for other, diverse, epithelial cell surfaces. Therefore, it is not unreasonable to expect that CD46 might not be available to serve as the initial, predominant pilin receptor on the mucosal surface of the human genital tract, the primary niche for gonococci. Support for this statement is derived from our previous data indicating that, at early time points post infection, CD46 is not required for gonococcal association with or invasion of primary, human, cervical epithelial cells (Edwards *et al.*, 2002).

The I (for inserted)-domain is an approximate 200 amino acid region found within the alpha subunit of some integrin heterodimer receptors. Our previous studies indicate that gonococcal pilus binds directly to the I-domain of CR3 and that this interaction is required for gonococcal adherence to and invasion of primary cervical epithelial cells (Edwards *et al.*, 2001, 2002). However, CR3 is not expressed by urethral epithelial cells (UECs) or by urethral...
tissue (Edwards et al., 2001). Host cell molecules available to serve as receptors for gonococcal pili have not been examined using primary, male UECs or urethral tissue. In addition to the leukocyte integrins (the \( \beta_2 \)-integrins, e.g. CR3), the \( \alpha_1 \)-integrins (the collagen receptor integrin \( \alpha_1 \beta_1 \), CD49a/CD29, very late antigen (VLA)-1) and the \( \alpha_5 \)-integrins (the platelet/collagen receptor integrin \( \alpha_5 \beta_1 \), CD49b/CD29, VLA-2) also contain an I-domain. Although not identical, a high degree of homology exists among the I-domains currently described (Fig. 1). Based on this high I-domain homology and the demonstration that gonococci bind to the I-domain of CR3, we reasoned that other I-domain-containing (IDC) integrins could potentially serve as pilus receptors and that some IDC integrins might be present on urethral epithelium. Collectively, our data demonstrated that while CD46 might be able to bind gonococcal pilus, IDC integrins appear to be preferentially used as the initial docking site for gonococci on primary, human, male urethral cells, on urethral tissue and on some immortal, human, epithelial cell lines. These studies suggest that the \( \alpha_1 \beta_1 \) and \( \alpha_5 \beta_1 \) integrins potentially serve as pilus receptors in vivo, initiating gonococcal adherence and colonization.

### Results

**Pili are required for the association of gonococci with primary male UECs**

Previous work performed in our laboratory demonstrates that invasion of primary, human, male UECs is mediated through the interaction of gonococcal LOS with the asialoglycoprotein receptor (Harvey et al., 2001). The work of several researchers indicates that pili are also required to initiate invasion of epithelial cell surfaces. Although it is generally accepted that these appendages are required for the association of gonococci with host epithelial cells, the host–gonococcal pilus interaction has not been examined using UECs. To confirm that pili are a necessary requirement to initiate infection of UECs, we first began our studies by quantifying the adherence of gonococci with these cells in the presence and absence of exogenous pilus competimers. The addition of 1 ng ml\(^{-1} \) of purified gonococcal pilus to association assays significantly impaired the ability of wild-type gonococci to associate with UECs (Table 1). Studies performed using a strain FA1090 pilE mutant, which exhibits a Pil\(^{+} \) phenotype, resulted in a similar finding with the association of mutant bacteria with UECs equivalent to only approximately 30\% of that observed for wild-type, FA1090, piliated, bacteria (14.1\% ± 0.8\%, wild type; 4.0\% ± 0.4\%, FA1090ΔPil).

### Table 1. Per cent association of *N. gonorrhoeae* strain 1291 with primary male urethral epithelial cells 30 min post infection.

| Competimer                        | Association (%) |
|-----------------------------------|-----------------|
| None                              | 12.49 ± 0.65    |
| rIl-domain                        | 4.20 ± 0.42     |
| FB12 anti-\( \alpha_2 \) mAb       | 2.90 ± 0.21     |
| P1H5 anti-\( \alpha_2 \) mAb       | 4.04 ± 0.19     |
| N-19 anti-CD46 pAb                | 11.58 ± 0.26    |
| H5A4 anti-CD11b                    | 12.96 ± 1.7     |
| Purified pil                        | 2.25 ± 0.17     |

Association assays were performed as outlined in the text. Values given are the mean values in which the per cent association was determined as a function of the original inoculum and the number of colony forming units formed with subsequent plating of the urethral epithelial cell lysates. Data given are the mean values obtained from at least three trials performed in triplicate. *P*-values were determined using a Kruskal–Wallis k-sample analysis of variance calculated for the association of gonococci in the presence of in comparison to the absence of the indicated, exogenous, I-domain competitors as outlines in the text. NA, not applicable; mAb, monoclonal antibody; pAb, polyclonal antibody.

I-domain-containing integrins are present on primary, male UECs

Two pilus receptors are described, CD46 (Källström et al., 1997) and CR3 (Edwards et al., 2002). Previous studies using primary, human, cervical epithelial cells indicate that gonococcal pilus can bind to the I-domain of CR3 (Edwards et al., 2002). \( \alpha_1 \) (CD49a) and \( \alpha_5 \) (CD49b) integrins also contain an I-domain. The high degree of homology existing between the I-domains currently described (Fig. 1) suggested that other IDC integrins could potentially serve as gonococcal pilus receptors. To determine whether IDC receptors are present within the male urethra we performed immunohistochemical analysis of sectioned, urethral tissue biopsies that were obtained from adult males. Confocal microscopy of immunolabelled tissue sections revealed that the \( \alpha_1 \)-, \( \alpha_5 \)- and \( \beta_2 \)-integrin subunits are present...
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present on the male urethra as indicated by the presence of a green fluorescence (Fig. 2). Fluorescence was visible on the apical, luminal, surface of the urethral epithelium; however, fluorescence was diffuse and did not appear to be polarized to a particular cell surface. Similar results were obtained for each of the antibodies used (i.e. anti-α1-integrin FB12, 5E8D9 and R-164; anti-α2-integrin N-19 and P1H5; and anti-β1-integrin antibodies). These data indicate that the collagen (α1β1, VLA-1) and the collagen/platelet (α1β1, VLA-2) receptors are potentially available for pilus binding during gonococcal disease. In contrast, CD46 was not readily visible in any of the urethral tissue sections examined with any of the antibodies used [i.e. E4.3, H-294, N-19 (Fig. 2)]. We obtained similar data when we repeated these studies using uninfected, primary, male UEC monolayers. α1, α2 and β1 integrins were readily visible; whereas, CD46 was not (data not shown). Studies using the H5A4 anti-CR3 monoclonal antibody (negative control) also failed to label male urethral tissue and UEC monolayers (data not shown).

Gonococcal pilus colocalizes with IDC alpha integrin receptors

To determine whether α1- and α2-integrins are capable of serving as receptors for pilus binding we incubated male urethral tissue sections with purified gonococcal pilus. Tissue sections were subsequently extensively rinsed before immunolabelling with the anti-pilus monoclonal antibody, IE8G8, followed by a second labelling procedure using an antibody to either the (A) anti-α1-integrin or (B) anti-α2-integrin antibodies revealed colocalization, as evidenced by the yellow fluorescence occurring from the combined signals of the red and green fluorophores. Only pilus was visible in tissue sections immunolabelled with an antibody to CD46 (C). No fluorescence was observed when both primary antibodies were excluded from the labelling procedure (D, negative control). Tissue specimens from three male donors were tested in these assays. Similar results were observed for all the antibodies used (see Experimental procedures) and for all tissue specimens. Shown are representative images of results obtained from the use of antibodies FB12 (anti-α1-integrin, A), P1H5 (anti-α2-integrin, B), and E4.3 (anti-CD46, C). Magnification: 40× plus 2× zoom.

Fig. 2. Expression of I-domain-containing integrins and CD46 on the urethral epithelium. (A) α1-integrin, (B) β1-integrin, (C) α2-integrin and (D) CD46. Tissue specimens from three male donors were used in these assays. Similar results were observed for all antibodies used (see Experimental procedures) and for all three tissue specimens. Shown are representative images of results obtained from the use of antibodies 5E8D9 (anti-α1-integrin, A), M-106 (anti-β1-integrin, B), P1H5 (anti-α2-integrin, C) and H-294 (anti-CD46, D). Magnification: A and B, 40×; C and D, 40× plus 2× zoom.

Fig. 3. Colocalization of gonococcal pilus with I-domain-containing integrins on male urethral epithelium. Purified pilus was used to overlay tissue sections as described in the text. A FITC-conjugated secondary antibody was used to visualize gonococcal pilus (green fluorescence); urethral cell constituents were visualized with a TRITC-conjugated secondary antibody (red fluorescence). Immunolabelling with anti-pilus and either of the (A) anti-α1-integrin or (B) anti-α2-integrin antibodies revealed colocalization, as evidenced by the yellow fluorescence occurring from the combined signals of the red and green fluorophores. Only pilus was visible in tissue sections immunolabelled with an antibody to CD46 (C). No fluorescence was observed when both primary antibodies were excluded from the labelling procedure (D, negative control). Tissue specimens from three male donors were tested in these assays. Similar results were observed for all the antibodies used (see Experimental procedures) and for all tissue specimens. Shown are representative images from assays obtained from the use of antibodies FB12 (anti-α1-integrin, A), P1H5 (anti-α2-integrin, B), and E4.3 (anti-CD46, C). Magnification: 40× plus 2× zoom.

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specificity of the assay (Fig. 3). These studies indicate that gonococcal pilus can associate with IDC integrins within the male urethra and that CD46 is not available to serve as a receptor on the male urethral epithelium.

It is generally accepted that the ability of pilus to initially bind to host receptors places the bacterium in proximity to the host cell surface, overcoming repulsive forces that would impede an intimate host-bacterial interaction (Ward and Watt, 1977; Wicken, 1985). Thus, the pilus–host receptor interaction occurs early in colonization of a cell surface. We examined UEC monolayers infected with gonococci for 0, 15, 30, 60 or 90 min by confocal microscopy. These studies demonstrated an early interaction occurring between gonococci and the α1- or α2-integrins. This is evidenced by the decreasing presence of yellow fluorescence (indicative of colocalization) with the increasing amount of time that gonococci were allowed to associate with UECs, as is shown in Fig. 4 at time points corresponding to 15, 30 and 90 min post infection. Colocalization of gonococci with IDC receptors appeared to peak at 30 min post infection (Fig. 4). However, by approximately 90 min post infection, little to no colocalization of gonococci with α1- or α2-integrins was observed (Fig. 4). Only GFP-expressing gonococci were visible in parallel (control) studies performed using an anti-αIIb-integrin antibody (which is not expressed by UECs) antibody or in which the primary antibody was omitted (Fig. 4). Similar results were obtained when Chang (Fig. 5) and ME180 cell lines were challenged with gonococci for variable time periods. Consistent with the data described above obtained from the

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pilus overlay assays, CD46 was only faintly visible on a very small subset of UECs, and its expression was not altered by gonococcal infection (Fig. 4). In contrast, CD46 was readily visible in Chang (Fig. 5), ME180 and T-84 (data not shown) immortal cell lines; however, we did not observe colocalization of gonococci with this cell surface constituent in these cells. In contrast to the studies described above, the presence of α1-, α2- or β1-integrins was not visible in uninfected T-84 cells, these receptors were only faintly visible after infection, and gonococci were not observed to colocalize with these IDC-integrins (data not shown). Collectively, our data are consistent with a role for IDC integrins in serving as pilus receptors on UECs.

I-domain competimers impair gonococcal adherence to primary UECs and to some immortal epithelial cell lines

Our previous studies indicate that at 30 min post infection gonococci are not internalized by UECs. Further data supporting a role for IDC integrins as receptors for gonococci are derived from competitive association assays in which gonococci were used to infect primary, male, urethral cells in the presence and absence of the I-domain competimers, rI-domain or anti-α1- or -α2 antibody (FB12 and P1H5 respectively). Although I-domain competimers severely impaired the ability of gonococci to associate with these cells, the addition of antibodies to CD46 and to CD11b (the CR3 alpha subunit, which is not expressed by urethral epithelium, negative control) did not significantly impair the ability of gonococci to associate with UECs (Table 2). These data are consistent with our previous published data demonstrating pilus-mediated adherence to the IDC integrin, CR3, on primary cervical epithelial cells and the negligible affect of CD46 antibody to gonococcal adherence on these same cells (Edwards et al., 2002).

Competitive association assays performed using Chang and ME180 immortal cell lines suggested that at 30 min post infection the presence of I-domain competimers inhibited the gonococcus association with these cells. In contrast to previous studies (Källström et al., 1997), we were unable to inhibit the association of gonococci with ME180 cells by the addition of anti-CD46 antibodies to our quantitative association assays (Table 2). Similarly, and consistent with our laser scanning confocal microscopy (LSCM) data described above, gonococci were not impaired in their ability to associate with T-84 cells with rI-domain or with any of the antibodies tested (Table 2). The addition of multiple I-domain competimers in a single assay performed using UECs or Chang cells demonstrated an additive effect in association inhibition (Table 3). Collectively, our data strongly indicate that IDC integrins are available to serve as pilus receptors on pri-
mary, human, epithelial cells. Our data also suggest that this phenomenon is not unique to primary cells in that IDC integrins might also serve as pilus receptors on some (e.g. Chang) but not all (e.g. T-84) immortal cell lines. Our data also hint at the existence of other, yet undefined, pilus receptors in that only approximately 20–40% adherence inhibition was observed in Chang and ME180 cells in the presence of the I-domain competimers used and at the indicated final concentrations.

Gonococcal pilus communoprecipitates with IDC integrins on male UECs but not T-84 cells

To further demonstrate that IDC integrins can serve as receptors for gonococcal pilus we performed immunoprecipitation of uninfected and 30 min-infected UEC and T-84 cell lysates (Fig. 6). Immunoprecipitation using the monoclonal antibody, IE8G8, specific for gonococcal pilus and Western blotting with an antibody specific for the α₁-integrin I-domain (antibody FB12) revealed the presence of an approximately 210 kDa band, confirming a pilus-α₁-integrin interaction occurred in UEC cells (Fig. 6A). Two additional peptide bands of a lower molecular mass were also observed and might be indicative of sample degradation. Alternatively, visualization of one of these two bands might be the result of antibody cross-reactivity, because the migration of this peptide was consistent with the molecular mass reported for the α₅-integrin (e.g. 160–165 kDa). An identical banding pattern was observed in an uninfected UEC cell lysate, which served as a positive control; however, no bands were observed in any of the lanes corresponding to communoprecipitates derived from uninfected cell monolayers or from assays performed with the omission of the primary or secondary antibody (negative controls) (Fig. 6A). Coimmunoprecipitation was not observed with studies performed using T-84 cells (Fig. 6A), which is consistent with data obtained from competitive association assays, and further suggest that in T-84 cells the I-domain does not appear to serve as an anchor for pilus adherence and that other pilus receptors probably exist on these cells.

The converse experiment, in which immunoprecipitation of UEC lysates was performed using anti-α₁-integrin, -α₂-integrin or -CD46 antibodies followed by probing Western Blots with the IE8G8 anti-pilus antibody, demonstrated the presence of a peptide band of an approximately 18 kDa in lanes corresponding to the anti-α₁- and -α₅ integrin immunocomplexes (Fig. 6B). These data are indicative of an association occurring between these IDC cell surface molecules and gonococcal pilus at 30 min post infection. A pilus-associated band was not present in lanes corresponding to assays performed using the anti-CD46 (N-19) antibody, providing further evidence that CD46 does not associate with gonococcal pilus at early time points (i.e. 30 min) post infection of UECs (Fig. 6B). A pilus–receptor interaction was not evident in uninfected cells. Similarly,
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(negative) control experiments, in which the primary or secondary capture antibodies were omitted, failed to show either the 210 kDa (α1-integrin) (Fig. 6A) or 18 kDa (pilus) (Fig. 6B) bands with subsequent Western blotting. Collectively, these studies clearly demonstrate gonococcal pilus associates with α1- and α2-integrins, but not CD46, on UECs.

α1-integrins coimmunoprecipitate with the asialoglycoprotein receptor H1 chain

The studies outlined above indicated that at 90 min post infection of UECs, only a small fraction of visible gonococci were observed to colocalize with α1- and α2-integrins. We also qualitatively observed that the presence of the α1-integrin on the UEC surface appeared to be decreased at 60–90 min post infection, upon comparison to earlier time points of infection (e.g. 30 min) (Fig. 4), perhaps suggesting their internalization with asialoglycoprotein receptor-mediated gonococcal invasion. Our previous studies have demonstrated that at 60 min post infection gonococci are just beginning to invade UECs; therefore, we would anticipate that the host cell constituents used for adherence and for invasion would both be engaged with gonococci at this point within our cell culture model of the infection process. To determine whether IDC integrins were internalized with gonococcal invasion we again performed immunoprecipitation studies. UECs were infected with gonococci for 60 min, and the infection was subsequently terminated by extensively rinsing and lysing cell monolayers. Cell lysates were then incubated with the IE9G9 anti-pilus, the (FB12) anti-α1-integrin I-domain, or the (N-19) anti-CD46 antibody. Western Blots were then probed for the presence of the asialoglycoprotein receptor within the immune complexes. The asialoglycoprotein receptor is a heterotrimer composed of two, 40–46 kDa, chains designated as H1, non-covalently associated with a single H2 chain of 43–50 kDa (Stockert, 1995). Membranes probed with the anti-asialoglycoprotein receptor monoclonal antibody, 6C2, revealed an approximate 40 kDa band consistent with the H1 chain of this receptor in samples corresponding to the α1-integrin receptor complexes (Fig. 7). Consistent with previously described data (Baricevic et al., 2002), a second band was also visible at approximately 80 kDa and may be indicative of H1 dimer formation (Fig. 7). The 40 and 80 kDa bands were also visible in an uninfected UEC lysate that served as a positive control. Peptide bands corresponding to the unglycosylated, immature, H1 chain were also visible in the urethral cell lysate (Fig. 7). No peptide bands were visible in immunoprecipitates derived from the use of the anti-CD46 antibody (Fig. 7), providing further support to the idea that CD46 does not play a role in adherence to the urethral epithelium. Peptide bands were also absent in lanes corresponding to anti-pilus immunoprecipitates indicating that the observed asialoglycoprotein receptor – α1-
integrin interaction was not the result of pilus binding to the asialoglycoprotein receptor, but rather was a direct association occurring between these two host cell surface molecules (Fig. 7). Consistent with our data described above peptide bands were not visible in lanes corresponding to samples to which the primary or secondary antibodies had been omitted (negative controls) (Fig. 7).

Discussion

Previous studies have eloquently demonstrated that pili, filamentous appendages extending from the surface of some bacteria, are a necessary component of the colonization process on mucosal surfaces. It is generally believed that initial, pilus-mediated, adherence of a bacterium to a host cell surface allows the organism to overcome the electrostatic repulsion occurring between negative charges on its own cell surface and that of its host cell (Ward and Watt, 1977; Wicken, 1985). This then allows the microorganism to form a subsequent, tighter, association with the target cell. In many instances this later association mediates internalization into the host cell, as is the case with *N. gonorrhoeae*.

The gonococcus is a strict human pathogen that uses variable mechanism of pathogenesis that are, in part, dependent upon the site of infection within its sole human host (reviewed in Jerse and Rest, 1997; Dehio *et al.*, 2000; Merz and So, 2000; Edwards and Apicella, 2004; Edwards *et al.*, 2004). Several receptors are described that mediate gonococcal internalization into a specific host cell. In addition, two pilus receptors are described. Work performed by Jonsson and coworkers identifies the complement regulatory protein, CD46, as the pilus receptor on ME180 cervical carcinoma cells (Källström *et al.*, 1997). A subsequent study performed by our laboratory identifies CR3 as the receptor used by gonococci to adhere to and to invade primary, human, cervical epithelial cells (Edwards *et al.*, 2002). These same studies negated a role for CD46 in adherence to and invasion of primary, human, cervical cells at early time points post infection (i.e. less than 3 h) (Edwards *et al.*, 2002), although CD46 clustering and its colocalization with gonococci do occur at later time points post infection (i.e. 6 h) (Edwards *et al.*, 2000), perhaps suggesting an alternative function for CD46 in cervical gonorrhoea.

Binding of gonococcal pilus to CR3 occurs through the CR3 α3 (CD11b) chain I-domain. Gonococcal porin and iC3b covalently linked to the lipid A core of LOS also bind to the I-domain and serve as secondary adhesins (Edwards and Apicella, 2002; Edwards *et al.*, 2002), collectively allowing a tight association with the cervical cell surface. Data supporting an I-domain-pilus interaction include the ability of ri-domain to bind to whole gonococci and to purified gonococcal pilus as determined by microscopy, ELISA, immunoprecipitation and Far-western Blot analyses, and the ability to inhibit gonococcal association
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with and invasion of primary cervical cells and CR3-expressing Chinese hamster ovary (CHO) cells with I-domain competimers (Edwards et al., 2001, 2002). The I-domain serves as the primary site for ligand binding by those integrin heterodimers that possess this inserted region, and binding of all known ligands to the $\alpha_\text{I} \beta_1$ and $\alpha_\text{II} \beta_1$ integrins occurs through their I-domain regions (Dickeson and Santoro, 1998). A high degree of sequence similarity exists among the I-domains currently described, and crystallography reveals that the major structural features of this region appear to be conserved (Dickeson and Santoro, 1998; Humphries and Newman 1998). It is well accepted that the I-domain configuration modulates integrin ligand binding, but also that the conformational state can vary with the particular cell type on which the integrin receptor is expressed (Zhang and Plow, 1996; Dickeson and Santoro, 1998; Li et al., 1998; Mesri et al., 1998; Xiong et al., 2000). Collectively, our data strongly indicate that the IDC integrins, $\alpha_\text{II} \beta_1$ and $\alpha_\text{IV} \beta_1$, serve as receptors for gonococcal pilus within the male urethra. Our ability to inhibit adherence to UECs and to some cell lines at 30 min post infection with anti-$\alpha_\text{II}$, $\alpha_\text{IV}$ or $\beta_1$ I-domain-directed antibodies and with R-domain suggest that it is this region within the $\beta_1$-integrin heterodimer that serves as the docking site for pilus binding. Colocalization of gonococci with IDC integrins was observed to be kinetically consistent with this role. Our data further suggest that IDC integrins may also serve as receptors on other tissues and other epithelial cell lines (Chang and ME180 cells).

As a first line of innate immune defence, the mucosal epithelium must be responsive to environmental changes. Although traditionally thought of as cell-adhesion molecules, integrins are capable of recognizing both soluble and cell-associated molecules and are exquisitely tuned to allow cells to rapidly adapt to changes within a given microenvironment. Epithelial cell integrins play diverse roles in modulating cell polarity, migration, proliferation, differentiation, survival, and inflammation (reviewed in Sheppard, 1996; Watt, 2002; Sheppard, 2003). Three populations of keratinocytes reside within the stratified epithelium: (i) stem cells (highly proliferative), (ii) transitional cells (limited proliferative capability) and (iii) committed cells (terminally differentiated, no proliferative capability) (Potten and Morris, 1988; Watt, 1989; Sheppard, 2003). As cells become differentiated they lose integrin function as well as integrin expression; consequently, cells that are closest to the basement membrane of the stratified epithelium express the highest levels of integrin receptors (Jones and Watt, 1993; Jones et al., 1995; Watt, 2002; Sheppard, 2003). Cells at the apical surface of the epithelium exhibit the lowest level of integrin receptors (Watt, 2002; Sheppard, 2003). However, integrin expression tends to be diffuse over the epithelial cell surface and is not necessarily confined to the basal surface of these cells (Watt, 2002; Sheppard, 2003; Sanders and Basson, 2004). Within the urethra, the mucosal epithelium is comprised of a single cell monolayer, which must be continually maintained and renewed through a process of co-ordinated terminal differentiation and apoptosis. This idea is consistent with the finding that rat urinary mucosa is predominately composed of transitional, proliferative or potentially proliferative, epithelial cells (Kaneko et al., 1984). Increased (diffuse) expression of $\alpha_\text{II} \beta_1$ and $\alpha_\text{IV} \beta_1$ integrins is observed with undifferentiated or proliferative epithelial cells (Lussier et al., 2000; Watt, 2002; Sanders and Basson, 2004), and these integrins are associated with the initiation of cell cycle progression and proliferation (Lussier et al., 2000; Sheppard, 2003). These data are consistent with our demonstration of diffuse $\alpha_\text{II} \gamma$, $\alpha_\text{IV} \gamma$ and $\beta_1 \gamma$ integrin expression within urethral epithelial tissue biopsies and UEC cell monolayers.

We were unable to demonstrate that any of the cellular constituents examined served as receptors for gonococcal adherence to T-84 cells, which form tight junctions and stratify in culture. Muza-Moons et al. (2003) showed that in T-84 cells $\beta_1$-integrins are primarily expressed on the basolateral surfaces, but also that infection with enteropathogenic Escherichia coli (EPEC) results in disruption of the epithelial integrity with the subsequent redistribution of $\beta_1$-integrins to the apical cell surface (Muza-Moons et al., 2003). Because we looked at gonococcal adherence at 30 min post infection when the epithelial cell monolayer is not compromised, one explanation for our inability to inhibit gonococcal adherence to these cells might be that the majority of $\beta_1$-integrins were localized to the basal cell surface and therefore were not available to serve as pilus receptors. Antibody-labelled $\alpha_\text{II} \gamma$, $\alpha_\text{IV} \gamma$ and $\beta_1 \gamma$ integrins were only faintly visible on the surface of T-84 cells by LSCM, which would tend to support this hypothesis. However, an alternate or additional explanation might be that on T-84 cells, those integrins that are apically expressed might exhibit an I-domain structural conformation that is not permissive for gonococcal adherence. Both of these hypotheses might be correct in that LSCM also did not reveal gonococcal colocalization with IDC integrins on T-84 cells. Regardless, neither of these statements negate the possibility that other as yet unidentified host cell surface constituent(s) exist for gonococcal pilus-mediated adherence. The addition of I-domain competimers to Chang and ME180 cell assays resulted in only approximately 20–40% adherence inhibition at the concentrations used. The addition of multiple I-domain competimers to a single assay using Chang cells further and significantly decreased gonococcal adherence but did not completely abolish it at the competimer concentrations used. These data hint at the presence of other pilus receptors on these cells. The addition of an anti-$\beta_1$ integrin antibody, which is

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directed at the I-domain binding pocket of the integrin heterodimer and therefore would inhibit adherence to both \(\alpha_\text{v}\)- and \(\alpha_\text{v}\beta_1\)-IDC integrins, reduced gonococcal adherence to UECs to levels comparable to that observed by the addition of exogenous purified pilus to adherence assays. The addition of multiple I-domain competitors to adherence assays only led to a moderate further decrease in gonococcal adherence compared with assays performed using a single competitor, perhaps suggesting receptor saturation, as adherence in these assays was also comparable to that observed by the use of the purified pilus competitor. Collectively, these data indicate that the \(\alpha_\text{v}\beta_1\)- and \(\alpha_\text{v}\beta_3\)-integrins serve as the primary receptors for pilus binding to UECs.

In contrast to previous studies described by Källström and coworkers (1997), we were also unable to inhibit the association of gonococci with ME180 cells by the addition of anti-CD46 antibodies to our quantitative association assays. We attribute this discrepancy to differences in the reagents and the procedures used. Most notably, we used a multiplicity of infection of 100 (vs. 200 described by Källström) and to ensure that our studies would reflect adherence and not adherence and invasion, we looked at time points corresponding to a 30 (vs. 90) min infection. Yet another important difference is that Källström et al. (1997) preincubated ME180 cell monolayers with 50 \(\mu\)g ml\(^{-1}\) of anti-CD46 antibody competitors for 1 h before the addition of gonococci to quantitative association assays, whereas we added 10 \(\mu\)g ml\(^{-1}\) assay competitors simultaneously with gonococci. Similarly, it is important to note that van Putten et al. (1998) were unable to inhibit the adherence of gonococci to Hep-2 (human larynx carcinoma) cells at 2 h post infection with the anti-\(\beta_1\)-integrin antibody, P4C10. However, OpaA-dependent, fibronectin-mediated invasion of Hep-2 cells was inhibited at 2 h post infection by P4C10 antibody addition (van Putten et al., 1998). This antibody recognizes a region comprised of amino acids 207 to 218 not associated with the I-domain binding pocket of IDC \(\beta_1\)-integrins (Takada and Puzon, 1993). We did not examine I-domain-mediated gonococcal adherence to Hep-2 cells and van Putten et al. (1998) did not look at early time points post infection; therefore, it can not be stated or negated that IDC integrins serve as pilus receptors on immortal, larynx epithelial cells. However, the additional ability of these researchers to inhibit gonococcal invasion of Hep-2 cells with a peptide containing the Arg-Gly-Asp (RGD) ligand, signature, integrin-binding motif (associated with non-IDC integrin ligands) argues against the IDC \(\beta_1\)-integrins in the described invasion process, but it does not exclude a role for IDC integrins in mediating an early, I-domain-dependent, pilus interaction on these same cells. Vitronectin internalization by integrin \(\alpha_\text{v}\beta_3\) occurs constitutively on U-251MG malignant astrocytes, conversely, \(\alpha_\text{v}\beta_3\)-mediated vitronectin internalization requires a signal from integrin \(\alpha_\text{v}\beta_3\) (Pijuan-Thompson and Gladson, 1997). Similarly, ligation of integrin \(\alpha_\text{v}\beta_3\) inhibits \(\alpha_\text{v}\beta_3\)-mediated phagocytosis but has no effect on \(\alpha_\text{v}\beta_1\)-mediated adherence (Blystone et al., 1994, 1995). These data provide precedent for the suggestion that on some cells and under certain circumstances integrin receptors may function cooperatively to independently mediate ligand adherence and/or internalization.

Several microorganisms use \(\beta_1\)-integrins as receptors for host cell adhesion and invasion. Engagement of \(\beta_1\) integrins by Yersinia enterocolitica (Schmid et al., 2004) and by Pseudomonas aeruginosa (de Bentzmann et al., 1996a) triggers interleukin (IL)-8 production and release. \(P.\ aeruginosa\) integrin-mediated adherence to the airway epithelium requires both integrin \(\alpha_\text{v}\beta_1\) and the gangliotetraosylceramide (asialo ganglioside M1, a\(\text{G}_{
abla}\)) receptor (de Bentzmann et al., 1996a, b; Roger et al., 1999). Integrin \(\alpha_\text{v}\beta_3\) is highly expressed in epithelia undergoing wound repair allowing this bacterium to become firmly established in the airway epithelium of cystic fibrosis patients (de Bentzmann et al., 1996b; Roger et al., 1999). Our data suggest that, within primary urethral cells, integrin \(\alpha_\text{v}\beta_3\) associates with the asialoglycoprotein receptor, which is consistent with the described role of integrins as costimulatory molecules (Ulanova et al., 2005). Although, to our knowledge, \(\beta_1\)-integrin-dependent IL-8 production by the urethral epithelium has not been demonstrated, engagement of the asialoglycoprotein receptor does trigger IL-8 production by the urethral epithelium, ultimately resulting in neutrophil influx (Harvey et al., 2002). Within the circulatory microenvironment neutrophils express little to no integrin \(\alpha_\text{v}\beta_1\) (Hemler, 1990; Werr et al., 2000). Upon extravasation, such as with inflammation, this integrin is dramatically upregulated and plays a critical role in neutrophil recruitment to extravascular tissues (e.g. the urethral epithelium) (Werr et al., 2000). Fibronectin is produced and secreted by these activated neutrophils (Werr et al., 2000). It is interesting to speculate that the gonococcus has adapted a mechanism of potentiating infection of the urethral epithelium by the concerted action of fibronectin, integrins and the asialoglycoprotein receptor.

Echovirus-1 (EV-1) is an enterovirus that can commonly cause conjunctivitis, meningitis, encephalitis, rash, and mild respiratory tract and enteric infections (Modlin, 2000a). Similar to what is observed for CR3 I-domain-mediated gonococcus adherence to and invasion of primary, cervical epithelial cells, invasion of human cells by EV-1 is mediated by two sites within the integrin \(\alpha_\text{v}\beta_3\)-I-domain (King et al., 1997; Sheppard, 2003; Pietiäinen et al., 2004). EV-1 is cytopathic in primate cell culture models but is not pathogenic in a mouse model (Modlin, 2000b). Eighty-four per cent identity exists between the
α2β1 I-domain found in humans and in mice (Pietäinen et al., 2004). Wild-type, murine, α2β1 integrin does not bind EV-1, but binding is conferred when the human α2 I-domain is inserted into a murine-human α2 chimera (Dickeson and Santoro, 1998). Additionally, EV-1 readily binds the α2β1 I-domain, but does not bind the I-domain of the α1 integrin subunit. Similarly, antibodies to the I-domain of the α2β1 and α2β2 integrins inhibit rotavirus adherence to epithelial cell monolayers; however, adherence is not inhibited when these same antibodies are used to inhibit binding to cells that are in suspension (Graham et al., 2003). These data have led to the idea that multiple structural features may contribute to I-domain binding and that differential ligand affinity exists for IDC integrins that are dependent upon the ligand and the cell type on which the integrin is expressed (Pietäinen et al., 2004). These complex structural determinants are thought to comprise interactive faces that cannot be reduced to short peptide motifs (Dickeson et al., 1999; Pietäinen et al., 2004). Recent evidence suggests that high affinity integrin function may require engagement of multiple binding sites that act synergistically (Obara et al., 1988; Aota et al., 1991; Bowditch et al., 1991, 1994; Kimizuka et al., 1991; Miyamoto et al., 1995; Loftus and Liddington, 1997; Humphries and Newman, 1998; Mesri et al., 1998). The only common element currently found among these synergistic binding sites is the presence of a surface-exposed flexible loop located between two β-sheet structures, a β-β-loop (Loftus and Liddington, 1997; Humphries and Newman, 1998; Kraus et al., 1998). A surface-exposed β-β-loop structure exists in gonococcal pilus (Smyth et al., 1996; Forest and Tainer, 1997; Forest et al., 1999) and may allow binding to IDC integrins. This idea may explain, in part, why it is possible for highly antigen variable microorganisms (e.g. piliated gonococci) to use different IDC integrins as pilus receptors on the same and on different cell types. Additionally, this might lend further support to the idea that on T-84 cells the integrin I-domain might not be in a conformation permissive for pilus binding.

The integrin–ligand association is thought to be transient, with ligand contact and release occurring as a cyclic phenomenon (Kishimoto et al., 1989; Arnaout, 1990; Zhang and Plow, 1996; Xiong et al., 2000). This characteristic makes integrin receptors ideally suited as pathogen docking sites in that they allow initial binding with subsequent intimate adherence mediated through multiple ligand interactions occurring at other sites within the integrin heterodimer (intraintegrin) or at sites on other host molecules (extra-integrin) that, in turn, mediate invasion. The gonococcus may have adopted both modes of integrin interaction as is evidenced by the ability of integrin receptors to mediate the adherence to and/or the invasion of gonococci to various human cells (Dehio et al., 1998; van Putten et al., 1998; Edwards et al., 2001). Several organisms have adapted methods to use IDC integrin receptors to initiate and to potentiate infection. Our previous studies have indicated that gonococci bind to the I-domain of CR3 to initiate adherence to and invasion of primary cervical epithelial cells. We have extended these studies to describe the interaction occurring between gonococcal pili and the I-domain of α2β1 and α2β2 integrins on the urethral epithelium. In contrast to the CR3-mediated gonococcal invasion of primary cervical cells, invasion of UECs is mediated by the asialoglycoprotein receptor. This might reflect minor structural differences existing between the CR3 and the α1 and α2 I-domains that do not allow porin binding; however, we have not examined a potential porin-I-domain interaction at the level of the urethral epithelium or with the β1 integrins. Because pilus expressed by the gonococcus and by the meningococcus exhibit a very high degree of similarity, it will also be interesting to determine whether these host cell molecules play a role in meningococcal disease as these integrins are present on platelets, neutrophils, and within the mucosal epithelium of the respiratory tract, suggesting that they might play a role in some of the clinical symptoms (e.g. thrombocytopenia and neutropenia) associated with severe meningococcal disease (Peters et al., 2001). Future studies will be aimed at answering these questions.

**Experimental procedures**

**Tissues and cell culture**

Urethra epithelia was obtained from adult males undergoing urologic surgery at the University of Iowa Hospitals and Clinics (Iowa City, IA) and was used to seed primary urethral cell culture systems as described by Harvey et al. (1997). Greater than one hundred urethral specimens have been collected to date, specimens from three separate patients were used to grow the primary UEC monolayers. Tissue specimens obtained from three additional patients were processed for cryosectioning. ME180 cells (ATCC # HTB-33) were cultured in McCoy’s 5 A medium (Life Technologies) according to ATCC recommendations. Chang (ATCC # CCL-20.2) and T84 (ATCC # CCL-248) cells were maintained in a 1:1 mixture of Dulbecco’s modified Eagle’s medium (DMEM) and Ham’s F12 medium supplemented with fetal calf serum (FCS) to a final 5% concentration (DMEM-F12 medium). ME-180 cells are derived from an omentum metastasis of a cervical carcinoma. Chang cells are a HeLa contaminant of conjunctiva epithelial cells. T84 cells are derived from a lung metastasis of a colorectal carcinoma. All of these cell lines are of human origin and are commonly used to study gonococcal pathogenesis. McCoy’s 5 A and DMEM-F12 media were replaced with antibiotic-free Defined Keratinocyte Serum Free Medium (dk-SFM) (Life Technologies, Rockville, MD) 48 h prior to infection studies. Surgical biopsies derived from the male urethra that were to be used for immunohistochemical tissue analyses were processed for cryosectioning as previously described (Edwards et al., 2000).
**Bacteria and infection studies**

*Neisseria gonorrhoeae* strains 1291 (Apicella, 1974; Dudas and Apicella, 1988), 1291-green, FA1090 (Cohen et al., 1994) and FA1090\(\alpha\)Pil (generously provided by J. Cannon, University of North Carolina, Chapel Hill) were used in the infection studies described below. *N. gonorrhoeae* strain 1291-green expresses green fluorescent protein (GFP) and was constructed by transforming strain 1291 with the GFP-encoding plasmid, pLES98, which was generously provided by V. Clark (University of Rochester, Rochester, NY). *N. gonorrhoeae* strains 1291 and FA1090 are clinically isolated gonococci. *N. gonorrhoeae* FA1090 is a serum-resistant, genital isolate from a patient with disseminated gonococcal infection. *N. gonorrhoeae* 1291 contains the pathogenicity island described by Dillard and coworkers (Dillard, 1999; Dillard serums-resistance and was isolated from a patient with gonococcal serum-resistant, genital isolate from a patient with disseminated gonococcal infection. *N. gonorrhoeae* 1291 exhibits moderate serum-resistance and was isolated from a patient with gonococcal urethritis. *N. gonorrhoeae* 1291 contains the pathogenicity island described by Dillard and coworkers (Dillard, 1999; Dillard and Seifert, 2001); however, FA1090 lacks this genetic island. The FA1090 Pil\(a\) mutant was constructed by deleting an approximate 250 base-pair region spanning the promoter and 5’ regions of *pilE*. Details concerning the construction of these bacteria will be described elsewhere. For infection studies bacteria were allowed to grow overnight (37°C, 5% CO\(_2\)) on GC-IsoVitalEx agar plates before harvesting with a sterile swab and resuspending in sterile physiological saline. Culture density was determined spectrophotometrically where an optical density of 1 at 600 nm was equivalent to 10\(^6\) bacteria ml\(^{-1}\). Bacterial cultures were further diluted in dk-SFM to a density of 10\(^5\) bacteria ml\(^{-1}\) and used to infect cell monolayers at a multiplicity of infection of 100. Infection was allowed to progress for variable time periods (as noted) after which the infection medium was removed and the cell monolayers were extensively washed with phosphate-buffered saline (PBS). Uninfected, control cell monolayers were simultaneously processed with challenged cell monolayers. Infected and uninfected (control) cell monolayers to be analysed by LSCM were fixed with 2% paraformaldehyde before immunolabelling as described previously (Edwards et al., 2000). Alternatively, infected and uninfected (control) cell monolayers were subsequently harvested for quantitative adherence (Edwards et al., 2000, 2001) or immunoprecipitation (Wen et al., 2000) assays as described previously. Based on our previous studies, infections for adherence assays were allowed to progress for only 30 min to ensure that data obtained were a measure of adherence rather than adherence and invasion.

**Immunolabelling and microscopy**

*Neisseria gonorrhoeae* strain 1291-green was used to challenge cell monolayers for variable time periods, as noted. Immunolabelling of frozen tissue sections and cell monolayers was performed as described by Edwards et al. (2000). Primary antibodies used for immunolabelling were specific for CD1b (H5A4 (Hildreth and August, 1985) (Developmental Studies Hybridoma Bank (DSHB), the University of Iowa), CD46 (E4.3, H-294 and N-19, Santa Cruz Biotechnology, Santa Cruz, CA); CD49a (integrin \(\alpha_1\)) (FB12, Chemicon International, Temecula, CA; 5E8D9, Upstate, Charlottesville, VA; R-164, Santa Cruz), CD49b (integrin \(\alpha_2\)) (N-19 and P1H5, Santa Cruz), integrin \(\alpha_{I\beta_2}\) (M-148, Santa Cruz), CD29 (b-integrin) (M-106, Santa Cruz), or gonococcal pilus (monoclonal antibody IE\(_G\), generously provided by Milan Blake, Food and Drug Administration (FDA), Bethesda (MD). Tetramethylrhodamine isothiocyanate (TRITC)- or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were applied to tissue sections, cell monolayers and purified gonococcal pilus, as noted. The ability of pilus to bind to urethral tissue was determined by incubating purified gonococcal pilus (a gift from M. Blake) with urethral cryosections. Frozen tissue sections were allowed to stand at room temperature (RT) for 30 min after which they were blocked (30 min, RT) with 50 mM glycine, rinsed thrice with PBS, and subjected to a second blocking step (1 h, RT) using 0.5% serum. Tissue sections on microscope slides were then flooded with 1 ng ml\(^{-1}\) purified gonococcal pilus and placed in a humidity chamber for 1 h at 37°C with gentle rotation. Following this incubation, slides were rinsed thrice with PBS and immunolabelled as described previously using the antibodies outlined above as specified. Immunolabelled tissue cryosections and cell monolayers were viewed using the Bio-Rad MRC-1024 Laser Scanning Confocal viewing system located at the Central Microscopy Research Facility at the University of Iowa (Iowa City, IA).

**Inhibition of *N. gonorrhoeae* attachment and invasion**

Primary, male, urethral; ME-180 omentum metastasis; Chang HeLa conjunctival contaminant, or T-B4 lung metastasis epithelial cell monolayers were challenged with 1291 or FA1090 wild type or FA1090\(\alpha\)Pil mutant gonococci as outlined above. Where indicated 1 ng ml\(^{-1}\) of recombinant (r)I-domain (generously provided by E. Brown, the University of California, San Francisco) or purified MS11 pilin competitor or 10 \(\mu\)g ml\(^{-1}\) of anti-\(\alpha_2\) (FB12), -\(\alpha_1\) (P1H5s), -\(\beta_1\) (M-106), -CD46 (N-19), -CR3 (HSA4) or pil (IE\(_G\)) antibody was included or excluded from inhibition assays. For experiments in which multiple antibodies were added to inhibition assays, 5 \(\mu\)g ml\(^{-1}\) of each antibody (i.e. FB12, P1H5s, and/or M-106) was used. Infected, control cell assays (devoid of rI-domain, pilus, or antibody competitors) were treated in parallel with infected experimental (including an adhesion competitor) inhibition assays. The association (i.e. adherence) of gonococci with host cells in the presence or absence of experimental additives was determined as a function of the original inoculum and the number of colonies formed with subsequent plating of the cellular lysate (Edwards et al., 2000). A Kruskal–Wallis non-parametric analysis of variance was used to determine the statistical significance of the association assays described above.

**Immunoprecipitation and western blot analyses**

Primary male urethral and immortal T-B4 epithelial cell monolayers were challenged with wild-type gonococci for 30 min. Uninfected and infected cell lysates were then subjected to immunoprecipitation as described previously using anti-pilus IE\(_G\), anti-\(\alpha_2\) (R-164), -\(\alpha_1\) (N-19) or -CD46 (N-19) antibodies. Wash steps were performed using stringent conditions. Infected, control cell lysates devoid of the primary or the secondary antibody were processed simultaneously with infected cell lysates to which both primary and secondary antibodies had been added. Following the final wash the immunoprecipitate was collected by low speed centrifugation (5 min, 2500 r.p.m.). The final pellet was resuspended in denaturing, electrophoresis buffer, boiled for 10 min, and separated on an SDS, 12% to 4% gradient, polyacrylamide gel. Separated immune complexes were then trans-
ferred to immobilon-P membranes (Millipore, Bedford, MA), and Western blotting was subsequently performed according to standard protocols using the anti-α integrin, 5E8D9 or FB12, anti-pilus IgE, or anti-asialoglycoprotein receptor (monoclonal antibody 6C2, DSHB, the University of Iowa) antibodies. Antibody-labelled constituents of collected immunocomplexes were detected with SuperSignal® West Pico Chemiluminescent Substrate (Pierce, Rockford, IL).

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Supplementary material

The following material is available for this article online.

Fig. S1. Sequence homology among the I-domains currently described.