The role of complement receptor 3 (CR3) in Neisseria gonorrhoeae infection of human cervical epithelia

Jennifer L. Edwards,1 Eric J. Brown,2 Kevin A. Ault3 and Michael A. Apicella1*
1Department of Microbiology, University of Iowa, Iowa City, IA 52242, USA. 2Department of Medicine, University of California, San Francisco, CA 94143, USA. 3Department of Obstetrics and Gynecology, University of Iowa, Iowa City, IA 52242, USA.

Summary
Neisseria gonorrhoeae is an important sexually transmitted pathogen and a major cofactor in HIV-1 infection. This organism uses different mechanisms to infect male and female genital tract epithelia. Receptor-mediated endocytosis of N. gonorrhoeae is the principle mechanism of entry into male urethral epithelial cells. Infection in men leads to a pronounced inflammatory response. In contrast, N. gonorrhoeae infection in women induces ruffling of the cervical epithelia, allowing a macropinocytic mechanism of entry. Infection in women is frequently asymptomatic, suggesting suppression of the inflammatory response.

N. gonorrhoeae-induced membrane ruffling and inflammation suppression are consistent with the ability of this bacterium to enter cervical epithelial cells, in vitro and in vivo, by interaction with complement receptor 3 (CR3), a receptor that does not trigger an inflammatory response. This receptor is present on cervical epithelial cells but not on male urogenital tract epithelia. N. gonorrhoeae engagement of CR3 initiates a unique mechanism of bacterial-induced membrane ruffling and internalization. These studies explain why the pathology of N. gonorrhoeae infection differs between males and females. Additionally, the observation that this receptor is present on cervical epithelia may provide insight into the pathogenesis of other sexually transmitted pathogens.

Introduction
Neisseria gonorrhoeae is the causative agent of the disease gonorrhea. In men, gonococcal infection develops as an acute urethritis, which is typically characterized by a purulent discharge that results as a consequence of the concurrent inflammatory response to infection. In women, gonococcal infection can develop as an ascending infection of the genital tract that can lead to an acute pelvic inflammatory disease, infertility or ectopic pregnancy.

The mechanisms by which the gonococcus infects and invades the female genital tract are only beginning to be understood. Research in our laboratory has shown that gonococci are capable of invading primary, human, epithelial cells derived from both the endo- and the ectocervix. These studies implied that the mechanism(s) used by the gonococcus to breach the cervical epithelium (Edwards et al., 2000) are distinct from those mechanisms used to invade the urethral epithelium in men (Harvey et al., 1997). Consistent with this idea is that >50% of women develop asymptomatic N. gonorrhoeae infection (Densen et al., 1982), suggesting suppression of an inflammatory response.

Phagocytosis that is mediated by complement receptor type 3 (CR3) occurs independently of a proinflammatory response in immune cells (Caron and Hall, 1998). CR3 exists as an integrin heterodimer composed of an alpha (αM or CD11b) and a beta (β2 or CD18) subunit. The primary ligand for CR3 is the complement (C') inactivation product, iC3b; however, this receptor exhibits broad ligand specificity. Several microorganisms bind CR3 to initiate infection (Cooper, 1991; Würzner, 1999). The direct association of CR3 with pathogenic Neisseria, however, has not been demonstrated. We describe here the occurrence of CR3 expression in primary, human, cervical epithelial cells and its co-localization with N. gonorrhoeae upon infection of these primary cells. We also describe the distribution of CR3 in immortalized tissue culture cell lines and within tissue biopsies derived from the male and female.
urogenital tracts. Monoclonal antibodies directed against CR3 inhibit gonococcal invasion of primary cervical cells and of CR3-transfected CHO cells. Similarly, *Clostridium* C3 neurotoxin inhibits gonococcal invasion. Further data supporting a role for CR3 in gonococcal infection of the cervical epithelium are obtained from immunohistochemical analysis of a clinical biopsy derived from a woman with culture-documented gonococcal cervicitis, which demonstrates gonococcal co-localization with CR3 in vivo. These data suggest that CR3 serves as a receptor for *N. gonorrhoeae* during infection of the cervical epithelium. Engagement of CR3 by the gonococcus or by anti-CR3 antibody results in membrane ruffling of primary endo- and ectocervical cells and CR3-tranfected K562 myeloid cells and CHO cells, suggesting that CR3 mediates [previously reported (Edwards et al. 2000)] N. gonorrhoeae-induced membrane ruffling. Collectively, these data may account for the pathological differences observed with *N. gonorrhoeae* infection of females in comparison with infection of males and identify a unique mechanism of bacterial-induced membrane ruffling.

**Results**

**Analysis of CR3 expression in primary human cervical epithelial cells**

Several lines of evidence suggest that C’ alternative pathway inactivation occurs with *N. gonorrhoeae* infection (Densen et al., 1982; Densen, 1989; Wetzler et al., 1992; de la Paz et al., 1995; Ram et al., 1998; 1999; Jarvis et al., 1999; McQuillen et al., 1999; Vogel and Frosch, 1999). This prompted us to examine the role of CR3 in *N. gonorrhoeae* infection of the cervical epithelium. Laser scanning confocal microscopy (LSCM) of cultured primary, human, ecto- and endocervical epithelial cells demonstrated the expression of both CD11b and CD18 (Fig. 1). Equivalent fluorescence was observed with either anti-CD18 (Santa Cruz Biotechnologies) or anti-CD11b (H5A4) antibodies, and a higher level of CR3 expression was observed in ectocervical cells compared with endocervical cells. Fluorescence was not observed with an IgG isotype control antibody. LSCM analysis of ecto- and endocervical cell infection studies using *N. gonorrhoeae* strains 1291, 1291-, MS11- and FA1090-green suggested that CR3 surface-level expression increased in the presence of *N. gonorrhoeae* (Fig. 2). In both ecto- and endocervical cells, co-localization of *N. gonorrhoeae* with CR3 was observed to occur by 30 min post infection (Fig. 2A and D). The *N. gonorrhoeae*-CR3 association continued to increase with 90 min (Fig. 2B and E) and 3 h (Fig. 2C and F) infections. These data are consistent with increased CR3 surface-level expression from intracellular stores and/or upregulation of CR3 production upon CR3 engagement by the gonococcus or by anti-CR3 antibody followed by the application of TRITC-conjugated secondary antibodies. CD11b (A and D) and CD18 (B and E) are visible in (uninfected) primary endo- and ectocervical cell greyscale images. Immunofluorescence was not observed with the use of an isotype control antibody (C and F).

G. Immunoprecipitation of primary ectocervical (lanes 3 and 4) and endocervical (lanes 5 and 6) cell lysates with the H5A4 capture antibody and subsequent Western blotting with anti-CD18 antibody revealed the presence of CD18 as an ~95 kDa band. H. Immunoprecipitation of ecto- (lanes 2 and 3) and endocervical (lanes 4 and 5) cell lysates with anti-CD18 capture antibody and subsequent Western blotting revealed CD11b at an ~170 kDa band, confirming CR3 expression. CD11b and CD18 were not observed using an isotype control antibody (lane G1) or with the omission of the primary capture antibodies from ectocervical (lanes G2 and H1) and endocervical (lanes G7 and H6) immunoprecipitation assays. Magnification: 60× oil: A–C, E and F; 40× oil: D.
Western blot analysis confirmed the presence of CR3 in primary cervical cells

To confirm the presence of CR3 in primary cervical epithelial cells, immunoprecipitation was performed using an antibody to CD11b or CD18 to capture CR3. Subsequent Western blot analysis using antibodies to CD18 or CD11b confirmed CR3 expression. Immunoprecipitation using the monoclonal antibody H5A4, specific for CD11b, and Western blotting with anti-CD18 antibody revealed the presence of an \( \approx 95 \) kDa band, consistent with CD18 (Fig. 1G). The converse experiment, in which immunoprecipitation was performed with anti-CD18 antibody and in which the Western blot was probed with H5A4, demonstrated the presence of an \( \approx 170 \) kDa band indicative of CD11b (Fig. 1H). Parallel immunoprecipitation and Western blot experiments using male urethral epithelial cells did not reveal the presence of either CR3 subunit (data not shown). Control immunoprecipitation experiments, in which the H5A4 or anti-CD18 capture antibody was omitted or an isotype control was used as the capture antibody, failed to show the 95 or 170 kDa bands with subsequent Western blotting (Fig. 1G and H).

Neisseria gonorrhoeae invasion of primary endocervical and ectocervical cells is dependent on CR3

To quantify the association of the gonococcus with CR3, we performed gentamicin resistance assays of infected endo- and ectocervical cells. Assays were performed in the presence or absence of antibodies to both the alpha and beta subunits of CR3 (Tables 1 and 2). The addition of anti-CD11b and anti-CD18 antibodies to the invasion assays resulted in >93% invasion inhibition of both ecto-and endocervical cells (Table 2). Invasion inhibition that occurred in the presence of the anti-CD18 antibody could be reversed by the addition (to the invasion assay) of a blocking peptide to this antibody. Similar infection studies,
which were performed with and without the gentamicin treatment, demonstrated that anti-CR3 antibodies inhibit gonococcal attachment to and subsequent invasion of primary ecto- and endocervical cells and CR3-transfected CHO (CHO-CR3) cells (Tables 1 and 2). Pretreatment of ecto- and endocervical cells with *Clostridium* C3 neurotoxin, which inactivates the effector domain of the Rho subfamily of GTPases, also significantly inhibited gonococcal invasion (Table 3), supporting a role for CR3-mediated phagocytosis, as C3-induced inhibition is characteristic of complement-mediated phagocytosis in immune cells (Caron and Hall, 1998). CHO-K1 cells, which do not express CR3, did not support *N. gonorrhoeae* adherence or invasion in the presence or absence of anti-CR3 antibodies or *Clostridium* C3 toxin.

Neisseria gonorrhoeae co-localizes with CD18 in vivo

The studies outlined above demonstrate that CR3 serves

### Table 1. Percentage inhibition of the association of *N. gonorrhoeae* with CR3 in the presence of anti-CR3 antibodies.

| Antibody competimer       | Cell type | H5A4 | Bear1 | IB4     | Anti-CD18 | α-CD18 + peptide |
|---------------------------|-----------|------|-------|---------|-----------|-----------------|
| Ectocervical              | 99.98 ± 0.0047 | ND   | ND    | 99.66 ± 0.0127 | ND    | ND            |
| Endocervical              | 99.55 ± 0.2627 | ND   | 98.64 ± 0.0106 | ND    | ND            |
| CHO-CR3                   | 99.22 ± 0.5619 | 98.16 ± 0.3938 | 97.51 ± 0.3960 | 95.09 ± 0.1896 | 22.68 ± 14.4064 |

Anti-CR3 antibodies inhibit the association of *N. gonorrhoeae* with CR3. Primary ecto- and endocervical, CHO-CR3 and CHO-K1 cell monolayers were pretreated with antibodies to the CR3 alpha (H5A4, Bear1) or beta (α-CD18, IB4) subunits before infection with *N. gonorrhoeae*, as indicated. Assays were performed at least three times, in triplicate, as described in the text. Anti-CR3 antibodies significantly inhibit *N. gonorrhoeae* association with and invasion of primary ectocervical cells, primary endocervical cells and CHO-CR3 cells. Gonococci did not associate with CHO-K1 cells. Values given were determined as a normalized function of the ability of the gonococcus to associate (i.e. adhere and invade) with cell monolayers in the presence, in comparison with the absence, of competitors. P-values were determined using a Kruskal–Wallis k-sample analysis of variance. ND, not determined.

### Table 2. Percentage inhibition of invasion of *N. gonorrhoeae* with CR3 in the presence of anti-CR3 antibodies.

| Antibody competimer       | Cell type | H5A4 | Bear1 | IB4     | Anti-CD18 | α-CD18 + peptide |
|---------------------------|-----------|------|-------|---------|-----------|-----------------|
| Ectocervical              | 99.81 ± 0.3771 | 99.29 ± 0.2370 | 99.65 ± 0.2489 | 98.71 ± 1.0062 | 19.24 ± 2.3040 |
| Endocervical              | 99.53 ± 0.4369 | 96.92 ± 2.8319 | 98.45 ± 1.3544 | 93.70 ± 6.4032 | 23.76 ± 3.0290 |
| CHO-CR3                   | 98.34 ± 0.2514 | 97.71 ± 0.5305 | 98.10 ± 0.2945 | 95.83 ± 1.4453 | 30.98 ± 2.6714 |

Anti-CR3 antibodies inhibit invasion of cell monolayers by *N. gonorrhoeae*. Primary ecto- and endocervical, CHO-CR3 and CHO-K1 cell monolayers were pretreated with antibodies to the CR3 alpha (H5A4, Bear1) or beta (α-CD18, IB4) subunits before infection with *N. gonorrhoeae*, as indicated. Gentamicin resistance assays were performed at least three times, in triplicate, as described in the text. Anti-CR3 antibodies significantly inhibit *N. gonorrhoeae* invasion of primary ectocervical cells, primary endocervical cells and CHO-CR3 cells. Gonococci did not invade CHO-K1 cells. Values given were determined as a normalized function of the ability of the gonococcus to invade cell monolayers in the presence, in comparison with the absence, of antibody competimers. P-values were determined using a Kruskal–Wallis k-sample analysis of variance.

### Table 3. Percentage invasion of *N. gonorrhoeae* in the presence of *Clostridium* C3 neurotoxin.

| Cell type | Ectocervical | Endocervical | CHO-CR3 | CHO-K1 |
|-----------|--------------|--------------|---------|--------|
| Without C3 toxin | 2.354 ± 0.2702 | 1.163 ± 0.9844 | 1.6802 ± 0.2605 | 0.0006 ± 0.0005 |
| With C3 toxin | 0.0945 ± 0.1306 | 0.0158 ± 0.0069 | 0.0104 ± 0.0054 | 0.0004 ± 0.0006 |

*Clostridium* C3 toxin significantly decreases *N. gonorrhoeae* invasion of ecto- and endocervical and CHO-CR3 cells. Cell monolayers were pretreated with *Clostridium* C3 toxin as described in the text before infection with *N. gonorrhoeae*. Values given were obtained from n = 3 trials performed in triplicate, in which the percentage invasion was determined as a function of the original inoculum and the subsequent colony-forming units with gentamicin treatment. P-values were determined using a Kruskal–Wallis k-sample analysis of variance.
**Fig. 3.** *N. gonorrhoeae* co-localizes with CR3 *in vivo*. Cryosections of a cervical biopsy obtained from a woman with culture-documented gonococcal cervicitis were immunolabelled with anti-CD18 (visible as a green fluorescence) and 2C3 (specific for the gonococcal H.8 outer membrane protein, visible as a red fluorescence). Co-localization of CR3 (green) with gonococci (red) occurs as a yellow fluorescence because of the combined signal of the two fluorophores. Presumed areas of co-localization are confirmed as a profile plot (see text), in which the line of the graph corresponding to the green channel (CD18) overlaps the line of the graph corresponding to the red channel (gonococci) as peaks within the graph.

A. Red channel (indicative of *N. gonorrhoeae*) of the image shown in (C).
B. Green channel (indicative of CD18) of the image shown in (C).
C. Merged image demonstrating co-localization as a yellow fluorescence.

D and E. Profile plot (E) of the vertical area corresponding to the red line in (D). The green line corresponds to the FITC-labelled CD18; the red line corresponds to the TRITC-labelled gonococci. Magnification: 63× oil with 5× zoom.

**Fig. 4.** LSCM demonstrates the distribution of CR3 within mucosal epithelia derived from the male and female reproductive tracts. Surgical tissue biopsies were processed for immunohistochemical analysis then immunolabelled with the anti-CD11b monoclonal antibody H5A4 (A–D and I–L) or anti-CD18 (panels E–H) followed by FITC-conjugated secondary antibodies and counterstaining with ethidium bromide. Arrows denote epithelial surfaces. CR3 is visible in ectocervical (A and E), endocervical (B and F), endometrial (C and G) and Fallopian tube (D and H) tissue biopsies as a green fluorescence. The absence of green fluorescence with LSCM analysis of anti-CD11b-immunolabelled vas deferens (I), male urethral (J), female urethral (K) and vaginal epithelia (L) demonstrates the lack of CR3 expression in these tissues. Green fluorescence observed in connective tissue is the result of autofluorescence. Magnification: 20×: A, C, E–G and I–K; 20× with 2× zoom: B, D and H.
as a receptor for gonococcal attachment and invasion of the cervical epithelium in vitro. To determine whether CR3 is bound by the gonococcus in vivo, we performed LSCM analysis of multiple sections of a cervical biopsy derived from a woman with culture-documented gonococcal cervicitis. Tissue sections were labelled with anti-CD18 and the monoclonal antibody 2C3, which is specific for the \textit{N. gonorrhoeae} H.8 outer membrane protein. Immunolabelling of these tissue cryosections revealed CD18 as a green fluorescence (Fig. 3B) and gonococci as a red fluorescence (Fig. 3A). \textit{N. gonorrhoeae} were found to co-localize with CD18, which was visible as a yellow fluorescence (Fig. 3C). Co-localization was confirmed as a profile plot in which the individual fluorescence of each fluorophore [within a designated (vertical) area of presumed co-localization; Fig. 3D] was recorded and plotted, individually, by the Zeiss 510 laser scanning confocal viewing system (Fig. 3E). LSCM profile plot analysis of five tissue sections demonstrated co-localization of 25 out of 27 (92.59\%) bacteria (or bacterial clusters) with CR3. These studies confirm in vitro studies using primary ecto- and endocervical cells and provide evidence that CR3 serves as a receptor for \textit{N. gonorrhoeae} infection in vivo.

Analysis of CR3 expression in tissue biopsies

Immunohistochemical analyses of surgical tissue biopsies derived from the male and female reproductive tracts were used to determine whether the \textit{N. gonorrhoeae}–CR3 association could account for the observed differences in the pathology of \textit{N. gonorrhoeae} infection between males and females. LSCM of surgical biopsies derived from the ectocervix, endocervix, endometrium and Fallopian tube revealed the presence of both alpha and beta subunits of CR3 (Fig. 4). Immunolabelling of tissue sections with anti-CD18 and anti-CD11b antibodies revealed comparable levels of immunofluorescence for each antibody in each of the tissues examined. CR3 expression appeared to be greatest in the ectocervix. Expression levels appeared to decrease progressively from the ectocervix to the upper female genital tract, with the lowest level of CR3 expression being observed in the Fallopian tube tissue. Examination of male urethra (Fig. 4I) and vas deferens (Fig. 4J) tissues failed to reveal the presence of either CR3 subunit. Similarly, tissue derived from the female urethral (Fig. 4K) and vaginal (Fig. 4L) epithelium did not express CR3. These data support the idea that a \textit{N. gonorrhoeae}–CR3
Engagement of CR3 stimulates membrane ruffling

The induction of membrane ruffles with *N. gonorrhoeae* infection of cervical, but not male urethral, epithelial cells has been reported (Edwards et al., 2000). We therefore performed microscopic analysis of gonococci-infected and -uninfected CR3-expressing cells to determine whether engagement of CR3 would elicit membrane ruffling. Extensive membrane ruffling of *N. gonorrhoeae*-infected K562-CR3, CHO-CR3 and primary cervical cells was observed by transmission electron microscopy (TEM), scanning electron microscopy (SEM; Fig. 5) and LSCM analyses. Ruffles were observed in the presence of gonococci, gonococci in the presence of anti-CR3 antibody or anti-CR3 antibodies without gonococci. Membrane ruffles were not observed on uninfected cells to which antibody had not been added or on cells incubated with an isotype control. Control assays using CHO-K1 cells, which do not express CR3, also failed to reveal the presence of membrane ruffles. These studies suggest that engagement of CR3 by the gonococcus or by anti-CR3 antibodies can initiate membrane ruffling.

Analysis of CR3 expression in immortalized epithelial cells

*Neisseria gonorrhoeae*-induced membrane ruffling has not been reported in immortal cell lines commonly used to study *N. gonorrhoeae*–host interactions; consequently, we examined immortal cell lines for CR3 expression. Infected and uninfected, cervical [HCK (Kiyono et al., 1998), End1 (Fichorova et al., 1997), ME180 (ATCC HTB-33)], endometrial (Hec1B) or E6/E7 transfected male urethral (UEC; to be described elsewhere) immortal cell lines did not express CR3, as determined by LSCM using anti-CD18 or H5A4 antibodies (data not shown). These data provide further support for CR3-mediated membrane ruffling with *N. gonorrhoeae* infection.

Discussion

We have described the distribution of CR3 in tissue biopsies derived from defined sites within the (human) male and female genital tracts and in primary, immortalized and malignant epithelial cells derived from these sites. LSCM demonstrated that CR3 was absent in tissues and cells derived from the male urethra and from tissue derived from the vas deferens, female urethra and vagina. CR3 was present on tissue and cells derived from the ecto- and endocervix and on endometrium and Fallopian tube tissue. Immortalized and malignant cell lines (i.e. HCK, End1, ME180, Hec1B and UEC) did not express CR3. *N. gonorrhoeae* appeared to induce upregulation of CR3 surface expression on primary ecto- and endocervical cells, in that gonococci were observed to co-localize with this receptor, and co-localization became increasingly prominent with extended infection. The association of the gonococcus with primary cervical cells and CR3-transfected CHO cells could be inhibited by the addition of anti-CR3 antibodies to quantitative infection assays. Gonococcal invasion of primary ecto- and endocervical cells was also inhibited by the addition of *Clostridium* C3 neurotoxin to invasion assays, which is consistent with CR3-mediated phagocytosis. Extensive membrane ruffling could be induced to occur in the absence of gonococci in primary endo- and ectocervical cells and in K562- and CHO-CR3 cells by the addition of anti-CD11b or -CD18 antibodies to infection assays. This suggests that the engagement of CR3 elicits membrane ruffling, which we have reported to occur previously in response to *N. gonorrhoeae* infection of the cervical epithelium (Edwards et al., 2000).

The role of C' in innate immunity is multifactorial; however, C' predominantly serves to eliminate foreign antigens and to regulate the inflammatory response directed towards these exogenous particles. C' protein C3 of the C' alternative pathway (AP) plays a paramount role in AP C' regulation, in that it serves to amplify the complement-mediated response by a positive feedback regulatory loop, which converts a relatively inefficient response into a highly efficient defence mechanism. Activation of the AP occurs constitutively at a low rate, which is tightly regulated by C' regulatory proteins, e.g. factors H (fH) and I (fI). Deposition of C3 on an exogenous surface (e.g. a bacterium) results in spontaneous C3 hydrolysis to produce C3b. C3b can bind factor B (fB) to generate C3 convertase activity, leading to the formation of the membrane attack complex and cell lysis. Alternatively, C3b can bind fH, leading to C' inactivation via cleavage of C3b by fI to produce iC3b, the primary ligand for CR3.

A number of microorganisms have adapted mechanisms not only to evade complement-mediated killing but also to pilfer C' components for their own advantage. Microorganisms that initiate infection via C' receptors frequently activate C', which subsequently results in C3 deposition on their cell surface (Hondalus et al., 1993). The effect of C' deposition is twofold: (i) it allows for evasion of immune surveillance; and (ii) it allows targeting to the appropriate host cell (Cooper, 1991). Microbial entry of host cells in a CR3 opsonic-dependent manner is thought to lead to a milder respiratory burst, thereby promoting increased intracellular survival (Mosser and Edelson, 1987; Würzner, 1999). Additionally, complement-mediated endocytosis occurs independently of a
proinflammatory response in immune cells (Caron and Hall, 1998).

Asymptomatic gonococcal urethritis develops in a small proportion of men. In contrast, 50–60% of women with gonorrhea initially exhibit asymptomatic infections, and 70% of women with disseminated gonococcal infection (DGI) lack symptoms of a genital tract infection (Densen et al., 1982). The ability of pathogenic Neisseria to cause the range of disease states associated with infection requires highly efficient methods of immune avoidance. Although strain-specific properties have been associated with resistance to complement-mediated killing (i.e. serum resistance) in vitro, most clinically isolated gonococci initially exhibit serum resistance (Densen, 1989; de la Paz et al., 1995; Ram et al., 1998; 1999; Vogel and Frosch, 1999). Ram et al. (1998) suggested that an increased conversion of C3b to iC3b on the gonococcal surface might contribute to serum resistance in vivo. This idea is supported by in vitro studies of gonococcal infection of neutrophils, in which a predominance of iC3b (in comparison with C3b) is found on the surface of gonococci (Jarvis et al., 1999; McQuillen et al., 1999; Vogel and Frosch, 1999). Conversion of C3b to iC3b on the gonococcal surface would permit efficient internalization of infecting gonococci (via CR3) into the cervical epithelium and could lead to the asymptomatic condition observed with gonococcal infection in a substantial proportion of women.

CR3 distribution is generally considered to be limited to immune cells (e.g. monocytes and neutrophils); however, CR3 is also found on renal glomerular (Sandilands and Whaley, 1985) and rectal (Hussain et al., 1995) epithelial cells. LSCM of surgical biopsies and of primary endo- and ectocervical cell monolayers (using two, well-defined antibodies to each CR3 subunit) demonstrated CR3 within the ectocervical, endocervical, endometrial and Fallopian tube epithelia; however, this expression appeared to decrease progressively from the ectocervix to the Fallopian tubes. Although CR3 is structurally and functionally related to the very late antigen (VLA) subfamily of integrins, which are present within the female genital tract (Sülz et al., 1998), these two distinct groups of proteins are not immunologically cross-reactive (Hynes, 1987). Additionally, isotype control antibodies failed to label primary cell monolayers or tissue cryosections. We therefore believe that our immunohistochemical data provide evidence for the presence of CR3 within the female genital tract. Furthermore, immunoprecipitation of primary cervical cell lysates confirmed the presence of CR3 within the ecto- and endocervix by the presence of the appropriate 95 kDa (CD18) and 170 kDa (CD11b) bands with subsequent Western blotting. These data suggest that the distribution of CR3 should now be extended to include the ecto- and endocervix and, possibly, the epithelia of the endometrium and Fallopian tubes.

The female reproductive tract and seminal fluid have been hypothesized to exhibit anomalous C' regulatory characteristics that exist to ensure successful reproduction by hindering an amplified immune response to seminal plasma (Vanderpuye et al., 1992). Full AP complement activity is reported in cervical mucous (Price and Boettcher, 1979; Vanderpuye et al., 1992); however, C4 of the complement classical pathway (CP) is only detected in a small subpopulation of luteal-phase cervical secretions (Vanderpuye et al., 1992). Collectively, these data suggest that the presence of CR3 within the female genital tract might function to eliminate exogenous antigens, after their inactivation in seminal fluid or cervical mucous, without a neutrophil influx.

Standard gentamicin resistance assays measuring gonococcal association with primary ecto- and endocervical cells in the presence of anti-CR3 antibodies demonstrated > 93% invasion inhibition with the antibody inhibitors used (Tables 1 and 2). LSCM analysis of multiple sections of a clinical biopsy derived from a woman with culture-documented gonococcal cervicitis further supports CR3-mediated N. gonorrhoeae cervical invasion. Confirmed CR3 co-localization of > 92% of the gonococci present in these tissue sections suggests that CR3-mediated gonococcal invasion exists as a primary mechanism of N. gonorrhoeae cervical infection in vivo.

Only a small proportion of total cellular CR3 is found on the surface of resting immune cells (Frank and Fries, 1991; Ram et al., 1998). CR3 activation causes the release of latent intracellular stores, which results in up to a 10-fold increase in CR3 surface expression (Kishimoto et al., 1989; Frank and Fries, 1991; Elemer and Edgington, 1994). Additionally, CR3 aggregation occurs in the early stages of phagocytosis (Kishimoto et al., 1989; Frank and Fries, 1991; Elemer and Edgington, 1994; Caron and Hall, 1998; van Kooyk et al., 1999). LSCM analysis of N. gonorrhoeae-infected primary ecto- and endocervical cells was reflective of the above-described events. Co-localization of infecting gonococci with CR3 was readily visible 30 min after infection of ecto- and endocervical cells, and this association became more pronounced by 90 min and 3 h after infection, suggesting an increase in surface-level expression of CR3. Additionally, co-localization of gonococci with CR3 was evident as clusters on the ecto- and endocervical cell surfaces.

SEM analysis demonstrated that the addition of anti-CR3 antibodies to K562- and CHO-CR3 cells and primary endo- and ectocervical cell monolayers resulted in membrane ruffles, suggesting that this phenomenon is elicited by CR3 activation. We reported previously that cervical infection by gonococci elicits membrane ruffling that is accompanied by a concentrated accumulation of the actin-associated
CR3 is present on the cervical epithelium (in which *N. gonorrhoeae* infection results in membrane ruffling and is frequently asymptomatic), but it is absent within the male urogenital tract (in which *N. gonorrhoeae* infection results in an inflammatory response and does not elicit membrane ruffling). We conclude that CR3-mediated invasion serves as a primary mechanism by which *N. gonorrhoeae* invades the cervical epithelium. The presence of CR3 on female genital, but not male urogenital, epithelia suggests that CR3 contributes to the high prevalence of asymptomatic *N. gonorrhoeae* infection in women. Furthermore, engagement of CR3 results in ruffling of the cervical epithelium by a process distinct from *Salmonella* - and *Shigella*-induced membrane ruffling.

### Experimental procedures

#### Tissues and cell culture

Surgical biopsies derived from the endo- and ectocervix that were used to seed primary cervical epithelial cell systems were procured and maintained as described previously (Edwards *et al.*, 2000) in defined keratinocyte serum-free medium (dk-SFM) (Life Technologies). Urethra epithelia was obtained from adult males undergoing urologic surgery at the University of Iowa Hospitals and Clinics (Iowa City, IA, USA) and used to seed primary urethral cell culture systems as described by Harvey *et al.* (1997). Primary male urethral cells were immortalized with the E6 and E7 genes from the human papilloma virus (to be described elsewhere) before use. E6/E7 immortalized human ectocervical keratinocytes (HCKs) and immortalized human endocervical (End1) cells were used to seed primary cervical epithelial cell systems as described by Harvey *et al.* (1997). 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Bacteria and infection studies

*Neisseria gonorrhoeae* strains 1291 (Apicella, 1974), 1291-green, FA1090-green and MS11-green were used in the infection studies described below. *Neisseria gonorrhoeae* strains 1291-green, FA1090-green and MS11-green express green fluorescent protein (GFP) and will be described elsewhere; the GFP-encoding plasmid, pLES98, was a gift from V. Clark (University of Rochester, Rochester, NY, USA). *Neisseria gonorrhoeae* 1291 and FA1090- and MS11-green parental strains [N. gonorrhoeae FA1090 (Cohen et al., 1994) and MS11 (Segal et al., 1985) respectively] are clinically isolated gonococci. *N. gonorrhoeae* FA1090 is a serum-resistant, genital isolate from a patient with disseminated gonococcal infection. *N. gonorrhoeae* 1291 is a serum-sensitive, urethral isolate obtained from a male patient with gonococcal urethritis. *N. gonorrhoeae* 1291, 1291-green and MS11-green contain the pathogenicity island described by Dillard (1999). For infection studies, bacteria were allowed to grow overnight (37°C, 5% CO₂) on GC-IsolVitaleX agar plates before harvesting with a sterile swab and resuspension in sterile physiological saline. Culture density was determined spectrophotometrically where an optical density of 1 at 600 nm was equivalent to 10^9 bacteria ml⁻¹. Bacterial cultures were diluted further in dk-SFM to a density of 10^7 bacteria ml⁻¹ and used to infect cell monolayers at a multiplicity of infection (MOI) 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 washed extensively with phosphate-buffered saline (PBS) before fixation with 2% paraformaldehyde. Uninfected, control cell monolayers were simultaneously processed with challenged cell monolayers. Infected and uninfected (control) cell monolayers were subsequently processed for LSCM, SEM or TEM as described previously (Edwards et al., 2000), or the cells were harvested for immunoprecipitation (Wen et al., 2000) assays before fixation.

**Immunolabelling and microscopy**

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 CD11b (H5A4, Hildreth and August, 1985) (Developmental Studies Hybridoma Bank (DSHB), the University of Iowa) and B1 (Immunotech, Marseilles, France)) or CD18 [anti-CD18 (Santa Cruz Biotechnology) and IB4 (Wright et al., 1983), generously provided by E. Brown (University of California)]. Tetramethyl rhodamine isothiocyanate (TRITC)- or fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were applied to cell monolayers and bacteria, as noted. Uninfected, tissue cryosections were labelled with FITC-conjugated secondary antibodies and counterstained with ethidium bromide (0.5 ng ml⁻¹ for 6 min). Clinical biopsy cryosections were incubated with 2C3 and anti-CD18 primary antibodies followed by immunolabelling with TRITC- and FITC-conjugated secondary antibodies respectively. The 2C3 monoclonal antibody recognizes the H.8 gonococcal surface protein. Infected and uninfected (control) K562 and K562-CR3 cells to be used for TEM analysis were labelled with colloidal gold secondary antibodies as indicated. Immunolabelled cryosections, cell monolayers and K562 cells were viewed using the Bio-Rad MRC-1024, the Zeiss 510 laser scanning confocal or the H-7000 (Hitachi) transmission electron viewing systems. Primary cervical cell and CHO cell monolayers that were processed for SEM analyses were viewed using the Hitachi S-4000 scanning electron microscope. All the microscopes used in these studies are located at the Central Microscopy Research Facility at the University of Iowa (Iowa City, IA, USA).

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

Primary cervical and CHO-CR3 and -K1 cell monolayers were pretreated (30 min at 4°C) with 20 μg ml⁻¹ H5A4, Bear1, IB4 or anti-CD18 antibody competitors before infection with gonococci as outlined above. Where indicated, anti-CD18 blocking peptide (Santa Cruz Biotechnology) was included in the inclusion assay. Infected, control cell assays (devoid of antibody competitors) and uninfected, control cell assays (with anti-CR3 antibodies) were treated in parallel with inhibition assays. The ability of gonococci to invade primary ecto- and endocervical cells and CHO-CR3 and -K1 cells was determined quantitatively by gentamicin resistance assays performed as described previously (Edwards et al., 2000), in which antibody competitors were included or excluded from the invasion assay as described above. Where indicated, primary endo- and ectocervical cell monolayers were pretreated (2 h at 37°C) with 10 ng ml⁻¹ *Clostridium* C3 neurotoxin before infection. Total association (i.e. adherence and invasion) of gonococci with primary endo- and ectocervical cells and CHO-K1 and -CR3 cells was quantified by the omission of gentamicin from the above-described invasion assay. Inhibition of gonococcal attachment and/or invasion was determined as a normalized function of the ability of gonococci to attach to and/or invade primary endo- and ectocervical cells or CHO cells in the absence of antibody inhibitors. 100% inhibition is the equivalent of no gonococcal adherence and/or invasion. 0% inhibition is the equivalent of the mean percentage association (adherence and invasion) or invasion for the endocervical (association 3.6390% ± 0.05076%; invasion 1.6153% ± 0.1741%), ectocervical (association 27.6289% ± 1.0281%; invasion 2.5868% ± 0.0594%) and CHO–CR3 (association 7.3064 ± 1.0885%; invasion 4.7875% ± 1.3911%) cells used. A Kruskal–Wallis non-parametric analysis of variance was used to determine the statistical significance of the association and invasion assays described above.

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