A co-operative interaction between *Neisseria gonorrhoeae* and complement receptor 3 mediates infection of primary cervical epithelial cells

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Summary

Little is known about the pathogenesis of gonococcal infection within the lower female genital tract. We recently described the distribution of complement receptor 3 (CR3) on epithelia of the female genital tract. Our studies further indicate that CR3-mediated endocytosis serves as a primary mechanism by which *N. gonorrhoeae* elicits membrane ruffling and cellular invasion of primary, human, cervical epithelial cells. We have extended these studies to describe the nature of the gonococcus–CR3 interaction. Western Blot analysis demonstrated production of alternative pathway complement components by ecto- and endocervical cells which allows C3b deposition on gonococci and its rapid conversion to iC3b. Anti-iC3b and -factor I antibodies significantly inhibited adherence and invasion of primary cervical cells, suggesting that iC3b covalently bound to the gonococcus serves as a primary ligand for CR3 adherence. However, gonococcal porin and pilus also bound to the I-domain of CR3 in a non-opsonic manner. Binding of porin and pilus to CR3 were required for adherence to and invasion of cervical epithelia. Collectively, these data suggest that gonococcal adherence to CR3 occurs in a co-operative manner, which requires gonococcal iC3b-opsonization, porin and pilus. In conjunction, these molecules facilitate targeting to and successful infection of the cervical epithelium.

Introduction

*Neisseria gonorrhoeae* is the aetiological agent of the disease gonorrhoea. In men gonococcal infection usually results in a profuse inflammatory response directed at infecting bacteria. In contrast, women who are infected with the gonococcus commonly exhibit asymptomatic infections, or they develop such minor symptoms that medical treatment is often not sought (Densen et al., 1982; Densen, 1989; Hook and Handsfield, 1999; Sparling, 1999). This asymptomatic condition is significant as it not only contributes to the persistence of the gonococcus within the general population, but it can also lead to more serious conditions, e.g. pelvic inflammatory disease (PID), infertility, ectopic pregnancy and disseminated gonococcal infection (DGI).

Recently, we described the distribution of complement receptor 3 (CR3) on epithelia of the female reproductive tract; however, we were unable to detect CR3 within the urogenital tract of men (Edwards et al., 2001). Our data suggest that engagement of this receptor serves as a primary mechanism by which the gonococcus is able to invade (human) ecto- and endocervical epithelial cells (Edwards et al., 2001). Our studies demonstrate that engagement of CR3 by the gonococcus results in membrane ruffling (Edwards et al., 2001) and bacterial entry via macropinocytosis (Edwards et al., 2000). In support of our *in vitro* studies, examination of clinical biopsies from women with gonococcal cervicitis revealed that *N. gonorrhoeae* co-localizes with CR3 *in vivo* (Edwards et al., 2001). Endocytosis that is mediated by CR3 occurs independently of a pro-inflammatory response in immune cells (Wright and Silverstein, 1983; Yamanoto and Johnston, 1984; Berton et al., 1992; Caron and Hall, 1998); consequently, invasion of the cervical epithelium via this receptor may be a factor in the asymptomatic nature of cervical gonorrhoea.

CR3 is an integrin heterodimer composed of an alpha (\(\alpha_m\) or CD11b) and a beta (\(\beta_2\) or CD18) subunit. Like other \(\beta_2\) integrins, the alpha subunit contains an I (also called A)-domain near its amino-terminus that is very important for ligand recognition (Dickeson and Santoro, 1998). The complement (C') alternative pathway (AP) C3b inactivation cleavage product, iC3b, binds to the I-domain and is
the primary ligand of CR3. This receptor exhibits broad ligand specificity. Several microorganisms can interact with CR3 (Cooper, 1991; Würzner, 1999); however, in most cases it is not clear if these interactions are I-domain-mediated. Although we have described CR3-mediated endocytosis as a primary mechanism by which the gonococcus is able to infect and invade the cervical epithelium (Edwards et al., 2001); whether this is a direct interaction with CR3, or whether it requires C′ deposition on the gonococcus, or whether this interaction is mediated through the CR3 I-domain remain to be elucidated. Several lines of evidence suggest that C′ activation followed by C3b inactivation may occur with N. gonorrhoeae infection (Densen et al., 1982; Densen, 1989; Rice, 1989; Wetzler et al., 1992; de la Paz et al., 1995; Ram et al., 1998a, 1998b; 1999; McQuillen et al., 1999; Vogel et al., 1999). We have therefore extended our previous studies to describe the gonococcus–CR3 interaction. We provide evidence that demonstrates production of alternative complement components by primary, human, ecto- and endocervical cells, which subsequently allows C3b deposition on gonococci and its rapid conversion to iC3b. Our data suggest that iC3b covalently bound to the gonococcus serves as a primary ligand for CR3 adherence; however, adherence to and invasion of cervical epithelia also requires binding of gonococcal porin and pili to the I-domain of CR3 in a non-opsonic manner. Collectively, these data suggest that opsonic and non-opsonic gonococcal adherence to CR3 occurs in a co-operative manner that facilitates targeting to and successful invasion of the cervical epithelium.

**Results**

**Primary human ectocervical and endocervical cells produce complement proteins**

Full AP C′ activity has been reported in cervical mucous (Price and Boettcher, 1979; Vanderpuye et al., 1992; Oglesby, 1998). To determine if the primary, human, ecto- and endocervical epithelial cell systems (used in these studies) produce AP C′ components, we performed Western blot analysis of (serum-free) tissue culture supernatants harvested from uninfected ecto- and endocervical cell monolayers. Western blotting of concentrated cervical cell supernatants indicated the presence of the C′ proteins factor B (fB) (Fig. 1A), properdin (Fig. 1B), factor H (fH) (Fig. 1C) and factor D (fD) (Fig. 1D) as bands of approximately 93 kDa, 56 kDa, 150 kDa and 24.4 kDa respectively. Factor I (fI) is synthesized as a preproprotein that undergoes extensive post-translational processing to form the mature serine protease. The presence of approximate 50 and 40 kDa bands upon Western blotting with polyclonal anti-factor I antibody indicated the presence of mature fi (Fig. 1E and F). Less prominent bands of approximately 25 and 15 kDa were also observed in endo- and ectocervical (Fig. 1F) supernatants and are consistent with unglycosylated fi polypeptide chains. C′ component C3 exists as an (approximately)
200 kDa protein composed of an alpha and a beta chain associated by a disulphide linkage. AP activation results in C3 cleavage to form C3a (9 kDa) and the 106 kDa (α) and 75 kDa (β) chains of C3b. Denaturing polyacrylamide gel electrophoresis followed by Western blotting with anti-C3 antibodies revealed bands of approximately 106 kDa and 75 kDa indicating the presence of C3 (Fig. 1G).

C3 cleavage products bind N. gonorrhoeae in vitro

AP inactivation is the result of fl-mediated cleavage of the 106 kDa C3 alpha chain, which renders C3 incapable of fB adherence. fl-mediated C3αc cleavage results in the formation of iC3b [68 (α1i or α′1i) and 40 (α2i or α′2i) kDa chains]; upon further cleavage of the iC3b (68 kDa) α1i chain, 33 kDa (C3dg) and 22.5 kDa fragments are produced. We performed Western blot analysis to determine if C3 opsonization of N. gonorrhoeae occurred in the primary ecto- and endocervical cell systems. Bacteria harvested from infection supernatants corresponding to 1, 2, 3, 5, 7 and 9 min of infection that were probed with anti-C3 antibodies revealed bands of approximately 106 kDa and 75 kDa indicating the presence of C3 (Fig. 1G).

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Fig. 2. iC3b opsonization of gonococci upon infection of primary ectocervical cells. Variable strains (as indicated) of N. gonorrhoeae were used to infect approximately 10⁵ primary ectocervical cells at an MOI of 100. An aliquot of the infection medium was removed at variable time points post infection and immediately processed for Western blot analysis as described in Experimental procedures. C3 cleavage products are indicated. The presence of C3b is indicated by 106 kDa and 75 kDa bands; iC3b by 75 kDa, 68 kDa, and 40 kDa bands. Further cleavage of the iC3b 68 kDa band results in C3dg formation as indicated by the presence of a 33-kDa fragment. A. C3 deposition on N. gonorrhoeae strain 1291 over the course of a 9-min infection (as indicated above each lane). Less than 1 min refers to the removal of an aliquot from the infection medium immediately after adding bacteria to primary ectocervical cell monolayers. B. The image given was obtained from a single blot; however, the centre portion (not relevant to this study) was removed. C3 deposition on N. gonorrhoeae strains VP-1 (lane 1), 24–1 (lane 2), FA1090 (lane 3), MS11 (lane 4), and FA1090Δpil (lane 5) at 5 min post infection of ectocervical cells.

Fig. 3. Western blot analysis for C3 presence in cervical secretions. Cervical secretions were collected from 18 women who were documented as having (+) or not having (–) N. gonorrhoeae cervicitis. Chemiluminescent detection of anti-C3 immunolabelled membranes was over extended to allow detection of peptide products potentially present at limiting concentrations. Arrows indicate the C3 cleavage products C3b-α, C3b-β, iC3b α1i, iC3b α2i, and C3dg (from top to bottom). *Samples collected at the time of menses.
Table 1. Anti-iC3b antibody-induced inhibition of gonococcal adherence to and/or invasion of primary cervical epithelial cells.

| Concentration (µg ml⁻¹) | % Inhibition of association | % Inhibition of invasion |
|-------------------------|----------------------------|-------------------------|
| 20                      | 96.0687 ± 2.91 (0.025)    | 96.9133 ± 2.43 (0.025) |
| 2                       | 88.0483 ± 6.03 (0.05)     | 93.7296 ± 2.17 (0.025) |
| 0.2                     | 71.7177 ± 0.06 (0.05)     | 71.5023 ± 4.82 (0.025) |
| 0.02                    | 53.6577 ± 1.13 (0.05)     | ND                      |
| 0.002                   | 34.5638 ± 3.36 (0.05)     | ND                      |

Values given were determined as a normalized function of the ability of the gonococcus to associate (i.e. adhere and/or invade) with or to invade primary ectocervical and endocervical cells in the presence of, in comparison to the absence of, an anti-iC3b antibody competitor as outlined in the text. 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 and are noted parenthetically. ND, not determined.

N. gonorrhoeae can bind the l-domain of CR3 in a non-opsonic manner

The above data indicate that iC3b opsonization exists as one mechanism by which N. gonorrhoeae is able to initiate infection of the cervical epithelia. However, these data do not exclude the possibility of a non-opsonic interaction of the gonococcus with CR3 present on the uterine cervix. To determine if the gonococcus can directly adhere to the l-domain of CR3 in the absence of iC3b, purified MS11 porin, MS11 or 1291 pili, LOS (of variable origin) and a N. gonorrhoeae strain 1291 cell suspension were separated by polyacrylamide gel electrophoresis and subjected to Far-Western blot analysis. Membranes were incubated with recombinant CR3 l-domain prior to probing with anti-

| µg ml⁻¹ | % Inhibition of association | Variance (P) | % Inhibition of invasion | Variance (P) |
|---------|-----------------------------|--------------|--------------------------|--------------|
| 10      | 98.9369 ± 0.15 (0.025)      | 98.7714      | 1.39 (0.025)             |
| 1       | 97.1983 ± 0.21 (0.025)      | 97.3605      | 1.41 (0.025)             |
| 0.1     | 96.6882 ± 1.04 (0.025)      | 98.6806      | 0.92 (0.025)             |
| 0.01    | 94.8515 ± 1.55 (0.025)      | 95.7580      | 2.23 (0.025)             |
| 10⁻²    | 88.2963 ± 2.33 (0.025)      | 74.0897      | 1.77 (0.05)              |
| 10⁻³    | 58.8888 ± 1.83 (0.05)       | ND           | NA                       |
| 10⁻⁴    | 26.5802 ± 1.99 (0.05)       | ND           | NA                       |
| 10⁻⁵    | 17.3563 ± 0.03 (0.05)       | ND           | NA                       |

Values given were determined as a normalized function of the ability of the gonococcus to associate (i.e. adhere and/or invade) with or to invade primary ectocervical cells in the presence of, in comparison to the absence of, an anti-fl antibody competitor as outlined in the text. Data given are the mean values obtained from at least four trials, which were performed in triplicate. P-values were determined using a Kruskal–Wallis k-sample analysis of variance and are not determined parenthetically. ND, not determined; NA, not applicable.

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his, -myc, or -I-domain (Bear1, LM2/1 and H5A4) monoclonal antibodies. Purified por and pil proteins bound CR3 rl-domain (Fig. 4A). We did not detect a direct association between LOS and rl-domain (data not shown). Observation of separated cell lysates revealed a predominant band of approximately 42 kDa and less prominent bands of slightly lower molecular mass. The 42 kDa polypeptide and a band corresponding to a molecular mass of approximately 20 kDa migrated with the same approximate molecular mass observed for the purified gonococcal porin and pilus respectively (Fig. 4A). Far-Western blot analysis of a panel of N. gonorrhoeae strains (including FA1090∆Opa and FA1090∆pil mutants) revealed similar patterns of I-domain adherence (Fig. 4A). The observed gonococcal non-opsonic interaction with rl-domain therefore is not a strain-specific property. However, rl-domain adherence to porin and pilin proteins was greater in the FA1090Opa mutant. A band of approximately 38 kDa to 40 kDa was identified as dimeric pilin by the absence of this band in the FA1090∆pil cell lysate, which was subsequently confirmed by stripping and re-probing Far-Western membranes with the anti-pilin monoclonal antibody IE8G8 (data not shown). To ensure that the observed protein bands corresponded to outer membrane constituents and not cytosolic factors that are geographically incapable of ligand function, outer membranes were isolated from N. gonorrhoeae strains 1291, FA1090 and MS11. Far-Western blot analysis of outer membrane preparations were consistent with those seen with analysis of whole cell lysates, indicating that the observed proteins correlate with putative CR3 ligands and are not artifacts of the assay used (data not shown). To further investigate porin as a putative ligand for CR3 I-domain we repeated Far-Western blot analysis (as described above) using a panel of N. gonorrhoeae isogenic por variants (Carbonetti et al., 1988; 1990). Strains FA19 (P.IA), MS11 (P.IB), FA19 expressing MS11 P.IB, MS11 expressing FA19 P.IA (Fig. 4B) and gonococci expressing PIA-P.IB hybrid porin of subclasses 1–9 bound rl-domain. ELISA confirmed these data in that microtitre plates coated with purified porin isolated from N. gonorrhoeae strains UU1 (P.IA) and MS11 bound rl-domain (Fig. 5) in a dose-responsive manner independently of porin isotype. Similar results were obtained when microtitre plates were coated with purified MS11 pil or with whole cell or outer membrane preparations from N. gonorrhoeae strains FA1090, MS11 or 1291 (Fig. 5). Collectively, these data demonstrate that the gonococcus can bind CR3 rl-domain in a non-opsonic manner, which occurs independently of strain variability.

Table 3. Inhibition of gonococcal adherence and/or invasion of ectocervical cells by rl-domain.

| µg ml⁻¹ | % Inhibition of association | Variance (P) |
|---------|-----------------------------|--------------|
| 1       | 98.5630                     | 0.37 (0.05)  |
| 0.1     | 96.7957                     | 0.21 (0.05)  |
| 0.01    | 92.2133                     | 1.04 (0.005) |
| 10⁻²    | 87.2361                     | 1.55 (0.005) |
| 10⁻³    | 77.5313                     | 2.33 (0.005) |
| 10⁻⁴    | 56.4501                     | 1.83 (0.05)  |
| 10⁻⁵    | 17.3816                     | 1.99 (0.05)  |

Values given were determined as a normalized function of the ability of the gonococcus to associate (i.e. adhere and/or invade) with primary ectocervical cells in the presence of, in comparison to the absence of, rl-domain competimer as outlined in the text. Data given are the mean values obtained from at least four trials, which were performed in triplicate. P-values were determined using a Kruskal–Wallis k-sample analysis of variance and are noted parenthetically.

Porin and pili are required for adherence to and invasion of primary cervical epithelial cells

CR3-mediated endocytosis serves as the primary mechanism by which the gonococcus infects and invades (primary) cervical epithelia; greater than 90% inhibition of adherence and invasion occurs when this interaction is blocked (Edwards et al., 2001). The above data indicate that porin and pilus mediate the interaction of the gonococcus with the I-domain of CR3. To examine more closely the role of these proteins in gonococcal infection of cervical epithelia, we performed quantitative inhibition assays (as described above) on the presence (or absence) of 3H1 (anti-por) or IE8G8 (anti-pil) antibodies, purified MS11 porin competimer, or by using the pil-deficient N. gonorrhoeae mutant, FA1090∆pil. Similarly, the role of opacity-associated (Opa) proteins were also examined through quantification invasion assays using the N. gonorrhoeae strain FA1090∆Opa. Anti-por as well as purified MS11 porin competimer significantly inhibited the interaction of the gonococcus with primary cervical epithelial cells (Table 4). Purified porin was observed to be a better inhibitor of gonococcal infection than the monoclonal anti-pil antibody, 3H1. Greater than 90% inhibition of adherence and invasion was observed at concentrations of 1 ng ml⁻¹ purified porin; 20 µg ml⁻¹ 3H1 (anti-por) antibody inhibited cervical epithelial association by approximately 86% (Table 4). In a similar manner, the addition of 20 µg ml⁻¹ anti-pil antibody to gentamicin-resistance assays resulted in significant inhibition (75.79% ± 0.65) of gonococcal invasion of primary ectocervical cells when compared with the absence of antibody. The plius-dependent decrease in the ability of the gonococcus to associate with primary cervical cells was even more pronounced in N. gonorrhoeae strain FA1090∆pil (Table 5). In contrast, no significant difference was observed in the ability of gonococci to adhere to and/or invade the primary cervical cells in the presence of an anti-CD46 antibody (Table 6). Similarly, adherence and invasion levels observed for N. gonorrhoeae FA1090∆Opa were comparable to its wild-
type parental strain, FA1090 (Table 5). Collectively, the above data indicate that both porin and pilus are required for the iC3b-mediated association of the gonococcus with CR3 on primary cervical epithelial cells. Opa proteins are not required for adherence or invasion of primary cervical epithelial cells, which supports our previous data (and more recently the data of Swanson et al. (2001)) obtained with N. gonorrhoeae Opa− phase-variants. The role of LOS in CR3-mediated endocytosis of the gonococcus by the cervical epithelium is examined in a separate study (Edwards and Apicella, 2002).

Discussion

We have provided evidence demonstrating that primary, human, ecto- and endocervical epithelial cells produce all the C′ components required for AP activation and inactivation. Neisseria gonorrhoeae infection of primary cervical cells resulted in C3b deposition upon the gonococcal surface that was rapidly converted to iC3b. Cervical secretions collected from women with gonococcal cervicitis or from women with normal gynaecological examinations supported these findings. Quantification association assays suggest that this bacterium appropriates C′ components for its own design in that anti-iC3b and anti-fl

Table 4. Effect of purified porin and anti-por antibody on gonococcal association with ectocervical cells.

| % Inhibition of association | Variance (P) |
|----------------------------|-------------|
| µg ml⁻¹ α-por antibody     |             |
| 20                         | 20.86 (0.01) |
| 2                          | 33.89 (0.025)|
| 0.2                        | 46.19 (0.025)|
| 0.02                       | 55.66 (0.025)|
| µg ml⁻¹ Por                |             |
| 10                         | 99.49 (0.05) |
| 1                          | 98.37 (0.05) |
| 0.1                        | 95.78 (0.05) |
| 0.01                       | 93.36 (0.05) |

Values given were determined as a normalized function of the ability of the gonococcus to associate (i.e. adhere and/or invade) with primary ectocervical cells in the presence of, in comparison to the absence of, an anti-por antibody or with purified MS11 porin competitor as outlined in the text. Data given are the mean values obtained from at least four trials, which were performed in triplicate. P-values were determined using a Kruskal–Wallis k-sample analysis of variance and are noted parenthetically.

Fig. 4. Far-Western analysis demonstrates gonococci bind CR3 I-domain. Purified gonococcal constituents or N. gonorrhoeae cell lysates that were transferred to a solid support medium were subjected to Far-Western blot analysis as outlined in the text.

A. The image given was obtained from a single blot; however, the centre portion (not relevant to this study) was deleted. Lanes: (1) MS11 porin (2) MS11 pil (3–7) Cell lysates of N. gonorrhoeae strain 1291 (3), MS11 (4), FA1090ΔOpa (5), FA1090 (6), and FA1090ΔPil (7).

B. Cell lysates of N. gonorrhoeae porin hybrids expressing either a PIA or PIB isotype. Strain MS11, PIB (lane 1); MS11 expressing FA19 porin, PIA (lane 2); FA19, PIA (lane 3); and FA19 expressing MS11 porin, PIB (lane 4).

Fig. 5. ELISA analysis of gonococcal adherence to recombinant I-domain. Microtitre plates were coated with purified gonococcal porin or pilus, gonococcal outer membranes (om), or whole N. gonorrhoeae cells as indicated and subjected to ELISA analysis as described in the text. Data given are the mean value obtained from microtitre wells corresponding to a 1/16 dilution of the origin concentration of I-domain (2 ng ml⁻¹) used. Mean values given were calculated from data obtained from at least three assays.

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antibodies or purified, recombinant, CR3 I-domain inhibited the ability of the gonococcus to infect and to invade primary cervical cells. We propose a co-operative mechanism of cervical cell adherence in which the direct interaction of porin and pilus with the CR3 I-domain augments the ability of iC3b-opsonized gonococci to bind to this receptor.

The primary site of C’ synthesis is the liver; however, increasing evidence demonstrates extra-hepatic production of some C’ proteins (Price and Boettcher, 1979; Barnum et al., 1992; Dovezenski et al., 1992; Gasque et al., 1992; Vanderpuye et al., 1992; White et al., 1992; Bischof et al., 1994; Hasty et al., 1994; Naughton et al., 1996; Springall et al., 2001). Production of some AP, but not classic pathway (CP), C’ components occurs within vaginal (Price and Boettcher, 1979) and endometrial epithelial cells (Barnum et al., 1992; Vanderpuye et al., 1992; Bischof et al., 1994; Hasty et al., 1994), and large amounts of C3 are produced by the uterus (Oglesby, 1998). Although full C’ activity has been demonstrated within cervical mucus, it is not clear if the presence of C’ in cervical mucus is the result of epithelial biosynthesis or transudation (Price and Boettcher, 1979; Vanderpuye et al., 1992). Analysis of concentrated, serum-free supernatants collected from primary, human, ecto- and endocervical epithelial cells revealed the presence of AP components (Fig. 1). In the absence of a (another) C’ source these data suggest de novo C’ biosynthesis by cervical epithelial cells. Additionally, AP C’ activity was observed within both primary cervical cell systems (used in these studies). Support for C’ activity is derived from quantitative gonococcal association/invasion assays performed in the presence of anti-iC3b neoantigen antibody and an anti-factor I antibody. Adherence and invasion of primary cervical epithelial cells by the gonococcus occurs through CR3 (Edwards et al., 2001), for which the primary ligand is iC3b. The anti-iC3b neoantigen antibody recognizes an epitope on C3 that is only present in its inactive form (i.e. iC3b) and the anti-fl antibody used in these assays specifically inhibits the serine protease activity of this enzyme, which is required for iC3b formation. Thus, inhibition of iC3b-gonococcus adherence to CR3 in the presence of these antibodies and in the absence of an alternative C’ source strongly infers that the observed AP activity was the result of (cervical) cellular biosynthesis. It is highly unlikely that surface-bound CD46 served as a receptor for the fl-mediated inactivation of C3b (bound to the gonococcal surface) in that iC3b-opsonization appears to be required for cervical cell adherence. Formation of iC3b on the gonococcal surface is therefore thought to occur in the fluid phase of the extracellular milieu. Additionally, our preliminary (unpublished) data and those of Oglesby (1998) indicate that expression of CD46 occurs primarily on the basolateral surface of the cervical epithelium. Furthermore, Tobaison and Seifert (2001) have indicated that an inverse correlation exists between CD46 expression and the ability of gonococci to associate with epithelial cells. Our inability to inhibit the association of gonococci with primary cervical cells in the presence of anti-CD46 antibody are consistent with these findings. These ideas are further supported by the presence of C3b and iC3b upon the gonococcal surface in Western blots of infection supernatants and which further demonstrates full AP activity in the primary cervical cell culture systems. Collectively, these data suggest that the cervical epithelium contributes to the C’ activity observed within cervical mucus and the lower female genital tract.

Table 5. Per cent adherence to and/or invasion of primary cervical cells by gonococcal pili and Opa deletion mutants.

| Strain of Gonococcus | Ectocervical Cells | Endocervical Cells |
|----------------------|-------------------|------------------|
|                      | Association (%)   | Invasion (%)     | Association (%) | Invasion (%) |
| FA1090 wt            | 16.1982 ± 3.20    | 1.7859 ± 0.13    | 12.4174 ± 0.80  | 1.0700 ± 0.24 |
| FA1090ΔOpa           | 17.7324 ± 1.83 (0.75) | 1.8318 ± 0.14 (0.025) | 12.2153 ± 1.40 (0.75) | 1.2043 ± 0.18 (0.05) |
| FA1090Δpil           | 3.9748 ± 0.08 (0.05) | 0.3847 ± 0.31 (0.025) | 0.3754 ± 0.04 (0.05) | 0.0513 ± 0.002 (0.05) |

Values given are the mean values in which the percent total association (adherence and/or invasion) and the percent invasion were determined as a function of the original inoculum and the subsequent number of colony forming units formed with subsequent plating of the ecto- or endocervical 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 mutant gonococcal strain in comparison to the wild-type strain.

Table 6. Per cent adherence to and/or invasion of primary cervical cells by gonococcal in the presence of anti-CD46 antibody.

|                      | Association (%) | Invasion (%) |
|----------------------|----------------|-------------|
| 1291 with α-CD46 Ab  | 30.3959 ± 2.69 | 2.8442 ± 0.55 |
| 1291 w/o α-CD46 Ab   | 26.2233 ± 1.9892 | 2.6249 ± 0.27 |
| P                    | 0.75            | 0.28         |

Values given are the mean values in which the per cent total association (adherence and/or invasion) and the per cent invasion were determined as a function of the original inoculum and the subsequent number of colony forming units formed with subsequent plating of the ectocervical 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 per cent gonococcal adherence and/or invasion in the presence of antibody in comparison to the absence of antibody.
The precise mechanism by which AP activation ensues is not fully elucidated. ‘Tickover’ in which continuous, low-level hydrolysis of C3 initiates AP activation is, currently, the prevailing hypothesis. Because spontaneous hydrolysis of C3 occurs constitutively, in a biological system there is always a supply of C3b present in circulation and the AP will always be present in a state of low-level activation. The chemical nature of a given particle’s surface dictates further AP amplification or its inactivation. Non-activating surfaces exhibit a higher affinity for fH than for fB; hence, C3b is rapidly inactivated to iC3b. Conversely, AP activators favour fB binding and, consequently, formation of the C3-convertase, C3bBb, ensues on the activating surface with concomitant C’ amplification. Resistance to C’-mediated killing (i.e. serum-resistance) therefore is not an all or none phenomenon but, rather, exists in variable degrees that are correlated with the affinity of a given particle for fH.

It is highly probable that the heterogeneity and complexity of the neisserial outer membrane allows C3b deposition on sites that are readily accessible to the actions of fH and fI as well as on protected sites that prohibit C’ inactivation. Analysis of gonococci isolated from infection studies at variable time points over a 9-min infection of ectocervical cells revealed a predominance of iC3b on the surface of gonococci by 1 min post infection (Fig. 2). We observed minimal variability between strains (expressing either a PIA or a PIIB isotype) in their ability to bind to and to inactivate C3b. Additionally, the presence or absence of sialic acid on the gonococcal surface does not influence the ability of this bacterium to bind and inactivate C3b or to invade primary cervical epithelial cells (Edwards and Apicella, 2002). These data would tend to suggest that factors other than sialylation state or porin isotype also contribute to surface-bound C3b inactivation within the microenvironment of the cervical epithelium.

In support of the above-described in vitro studies, iC3b (in comparison to C3b) was readily detected in six of the seven cervical secretion specimens collected from women with gonococcal cervicitis. Cervical mucus is estimated to contain slightly greater than one-tenth of the C’ activity observed for human serum (Price and Boettcher, 1979; Vanderpuye et al., 1992). We were unable to detect C3b and iC3b in secretion specimens collected from women with normal (uninfected) gynaecological exams. We observed for human serum (Price and Boettcher, 1979; Armaout, 1990; Xiong et al., 2000; Zhang and Plow, 1996). Additionally, it is well accepted that the configuration of the l-domain modulates ligand binding (Zhang et al., 1996; Dickeson and Santoro, 1998; Li et al., 1998; Mesri et al., 1998; Xiong et al., 2000). Recent evidence suggests that high affinity integrin function may require engagement of multiple binding sites that are intimately linked, act synergistically and are under allosteric control (Obara et al., 1988; Bowditch et al., 1991a, 1991b; Kimizuka et al., 1991; Aota et al., 1994; Miyamoto et al., 1995; Loftus and Liddington, 1997; Humphries and Newman, 1998; Mesri et al., 1998). The only common element currently found among these short (consisting of a few amino acids) synergistic recognition sites is the presence of a flexible loop located between two β-sheet structures (Loftus and Liddington, 1997; Humphries and Newman, 1998; Kraus et al., 1998). Gonococcal porin (van der Ley et al., 1991; Rudel et al., 1996; Cooke et al., 1998) and pilin (Symth et al., 1996; Forest and Tainer, 1997; Forest et al., 1999) both contain surface-exposed β-β-loop structures that may allow binding to the CR3 l-domain.

Gonococcal porins are voltage-gated channels that exhibit slight anion selectivity; however, upon their insertion into an eukaryotic (host) cell membrane an ATP or GTP regulated (Rudel et al., 1996) shift in porin voltage-dependence is observed and is associated with a calcium flux (Young et al., 1983; Rudel et al., 1996). In addition to porin, gonococcal pilin also induces a calcium flux within eukaryotic cells (Källström et al., 1998). Chemically blocking the release of intracellular calcium stores induced by pilus results in the inability of the gonococcus to form a tight (secondary) association with its host cell and decreased gonococcal adherence (Källström et al., 1998). Functional modulation, the process of qualitative receptor remodelling (Hughes and Pfaff, 1998), is a dynamic process that is dependent upon and is regulated by changes in membrane potential and in intracellular calcium concentrations that are governed by voltage-gated channels (Alteri et al., 1990). fMLP, A/GTP, A/GDP (Brown, 1991) and ionophores can elicit CR3 functional modulation and...
the ensuing intracellular calcium flux is thought to initiate signal transduction cascades essential to affinity changes (Alteri and Edgington, 1988; Alteri et al., 1990). Calcium flux in response to functional modulation is biphasic, exhibiting an early transient calcium flux that is independent of extracellular calcium stores and a second, more prolonged and sustained, calcium peak that requires extracellular calcium (Alteri et al., 1990).

In view of these data it can be speculated that pilin- and porin-mediated binding of the gonococcus to CR3 may elicit a biphasic calcium flux in cervical epithelial cells. An initial interaction of pilus with the CR3 I-domain could cause an (early) increase in intracellular calcium concentration from intracellular calcium stores. Pilus binding could then allow a subsequent interaction between porin and the CR3 I-domain that may trigger a second calcium flux and increase in the affinity of CR3 receptors for iC3b deposited on the bacterium's surface. A similar idea has been proposed for fibronectin binding to integrin receptors in which a hierarchy of five or more adhesive ‘systems’ (i.e. domains) within the fibronectin molecule function independently but also work co-operatively to mediate integrin binding (Humphries et al., 1987; Obara et al., 1988; Aota et al., 1991; Nagai et al., 1991; Kimizuka et al., 1991). Our unpublished and previously published data demonstrates that in the absence of calcium the ability of gonococci to invade primary ecto- and endocervical cells is obliterated (Edwards et al., 2000). Further evidence for this hypothesis can be derived from the ability of porin to inhibit fMLP-induced signalling events in neutrophils (Haines et al., 1988; 1991; Bjerknes et al., 1995), suggesting these molecules share effector proteins. Further examination will be required to definitively elucidate the roles of porin and pilin in CR3 functional modulation and in iC3b-mediated gonococcal adherence to this receptor.

The interaction of the gonococcus with its sole human host is complex and multifactorial. Studies focusing on neisserial serum-resistance have primarily involved cell-free systems in which serum-resistance has been measured as the ability of these organisms to survive the lytic action of pooled human serum. However, mucosal membranes serve as the primary site for neisserial infection. Increasing evidence suggests that AP C′ components are produced by epithelial cells, albeit at a much lower level than is observed in human serum. iC3b-mediated adherence to CR3 does not result in a respiratory burst (Wright and Silverstein, 1983; Yamamoto and Johnston, 1984; Berton et al., 1992; Caron and Hall, 1998) and therefore may provide a mechanism by which the gonococcus is protected from the harsh extracellular environment of the cervix and, consequently, allow colonization. These ideas are consistent with the wide variability associated with the incubation period, the clinical manifestations and the high

Fig. 6. A model of the co-operative interaction between the gonococcus and the I-domain of CR3. This figure demonstrates the model that we propose for the co-operative opsonic and non-opsonic interactions that occur between N. gonorrhoeae and the I-domain of CR3. We have shown that iC3b bound to the surface of the gonococcus (opsonic) in conjunction with the direct adherence of gonococcal porin and pilus (non-opsonic) to the I-domain of CR3 are required for adherence to and invasion of primary, human, cervical epithelial cells. The binding of gonococcal pilus to the I-domain of CR3 allows the gonococcus to overcome the electrostatic repulsion of negative charges between the cervical cell and its own cell surface (non-opsonic interaction). Deposition and inactivation of C3b (forming iC3b, the primary ligand for CR3) on the gonococcus surface spatially augments the direct interaction of porin with the CR3 I-domain. The binding of gonococcus-bound iC3b, porin and pilus to the CR3 I-domain results in a high affinity, specific interaction between the gonococcus and the cervical epithelium. Inhibition experiments indicate that both opsonic and non-opsonic interactions are necessary for gonococcal adherence to and invasion of cervical epithelial cells.
Experimental procedures

Cell culture and cervical secretion samples

Surgical biopsies derived from the ecto- and the endocervix 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, Rockville, MD). The dk-SFM was replaced with dk-SFM lacking antibiotics at least 24 h before infection studies. For analysis of complement production by ecto- and endocervical cells, primary cervical cell monolayers were incubated with dk-SFM for greater than 12 h, after which the medium was removed and concentrated (10-fold) by centrifugation through centricon-3 filter units (Millipore, Bedford, MA) and resuspension in 0.1 M Tris (pH 7.5). Clinical, cervical secretion, swab samples were collected from 18 women, 11 of whom were negative for N. gonorrhoeae infection (normal, uninfected) and 7 of whom were documented as having a N. gonorrhoeae infection. The clinical cervical secretion samples were generously provided by K. A. Ault (University of Iowa, Iowa City, IA) and B. Vanderpohl (Indiana University, Indianapolis, IN).

Bacteria and infection studies

Neisseria gonorrhoeae strains 1291 (Apicella, 1974; Dudas and Apicella, 1988), 24-1 (Griffiss et al., 1987), FA1090 (Cohen et al., 1994), FA1090-o3Pa, FA1090-spI (Cohen and Cannon, 1999), MS11 (Schoolnik et al., 1984; Segal et al., 1985), FA19 (Stephens and Shafer, 1987; Carbonetti et al., 1990; van Putten et al., 1998) and VP-1 (Makino et al., 1991; van Putten et al., 1998; Lorenzen et al., 2000) were used in the infection studies described below. These bacteria are clinically isolated gonococci that vary in their resistance to complement-mediated killing (i.e. serum-resistance). Neisseria gonorrhoeae FA1090 and VP-1 are serum-resistant, genital isolates from patients with disseminated gonococcal infection. Neisseria gonorrhoeae 1291 exhibits moderate serum-resistance and was isolated from a patient with gonococcal urethritis. Serum-sensitive gonococcal isolates, 24-1 and MS11, were obtained from patients with pelvic inflammatory disease. Neisseria gonorrhoeae 1291, 24-1 and MS11 contain the pathogenicity island described by Dillard (1999); however, FA1090 and VP-1 lack this genetic island. FA1090 o3Pa and PiIE deletion mutants were constructed according to the method of Johnston and Cannon (1999). These bacteria will be described elsewhere. For infection studies bacteria were allowed to grow overnight (37°C, 5% CO2) 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 107 bacteria ml−1. Bacterial cultures were further diluted in antibiotic-free dk-SFM to a density of 101 bacteria ml−1. Bacteria (107 ml−1) were then 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 extensively washed with phosphate-buffered saline (PBS). Uninfected, control cell monolayers were simultaneously processed with challenged cell monolayers. Infected and uninfected (control) cell monolayers were subsequently harvested for quantitative association (i.e. adherence and invasion) or invasion assays. Alternatively, (for determination of C3 opsonization), aliquots of infection supernatants were harvested at various time periods post infection (as noted), immediately transferred to ice (to inhibit C′ enzymatic activity) and bacteria were collected by centrifugation (4°C, 4000 r.p.m., 5 min). Bacteria were rinsed three times (on ice) with sterile physiological saline before resuspension in a 0.1 M Tris-0.1% SDS solution.

Western blot analysis for complement production and deposition

Western blotting was performed using standard protocols. Where indicated, N. gonorrhoeae (collected from infection supernatants) cell lysates, concentrated cervical cell culture supernatants, or cervical secretion samples were separated on 4% to 12% denaturing polyacrylamide gradient gels. Western blotting was performed with polyclonal anti-C3 (Atlantic Antibodies, Scarborough, ME; Quidel, San Diego, CA) or -fI (Quidel) antibodies or with monoclonal antibodies specific for fI (Quidel), fB (Quidel), fD (The Binding Site, Birmingham, UK), properdin (Quidel), or fH (Quidel), as noted.

Inhibition of N. gonorrhoeae adherence and invasion

Primary cervical cell monolayers were infected with gonococci as outlined above. Variable concentrations of monoclonal anti-fI, anti-iC3b neoantigen (Quidel), 3H1, or iEG9 antibody or recombinant, murine, CR3 I-domain (rI-domain) or purified MS11 P .I.B porin competitors were simultaneously added with gonococci as noted. Alternatively, 20 μg ml−1 anti-CD46 monoclonal antibody (E4.3, Santa Cruz Biotecnology) was added to cervical cell monolayers with gonococci. Infected, control cell assays (devoid of antibody, porin, or fI-domain competitors) were treated in parallel with inhibition assays. The monoclonal anti-factor I antibody used in these studies inhibits fI enzymatic activity. Antibodies 3H1 and iEG9 recognize conserved epitopes on gonococcal porin and pilus respectively. The fI-domain contains both a histidine and a c-myc tag and its detailed construction will be described elsewhere. Briefly, a 187 amino acid (AA 145–322) segment of murine Mac-1 (i.e. CD11b/CD18) was cloned into pPICZalpha (Invitrogen, Carlsbad, CA). Protein production was induced in pPICZalfpha-transformed Pichia pastoris by a 24 h incubation in 0.5% methanol following which the supernatant was collected and the fI-domain was purified on a Ni2+ column. The fI-domain purity was assessed by silver-staining SDS polyacrylamide acrylamide gels in which the column eluant was separated. The ability of
gonococci to invade primary ecto- and endocervical cells was quantitatively determined using standard gentamicin-resistance assays, performed as described previously (Edwards et al., 2000) and in which competitors were included or excluded from the invasion assay as described above. The total association (i.e. adherence and invasion) of gonococci with primary ecto- and endocervical cells was quantified by the omission of gentamicin from the above described invasion assay. Per cent adherence and/or invasion were determined as a function of the original inoculum and the number of colonies formed with subsequent plating of the cellular lysate. 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 in the absence of competitor inhibitors. A Kruskal–Wallis non-parametric analysis of variance was used to determine the statistical significance of the association and invasion assays described above.

**Determination of CR3 I-domain adherence to N. gonorrhoeae**

Overnight cultures of *N. gonorrhoeae* strains 1291, FA1090, FA1090ΔOpa, FA1090Δpil, VP-1, 24-1, MS11, FA19, or a panel of *N. gonorrhoeae* FA19 and MS11 porin variants (Carbonetti et al., 1988; 1990) were harvested as described above and resuspended in sterile physiological saline. Neisseria gonorrhoeae whole cell lysates or outer membranes were separated on a denaturing, 4% to 12% gradient, polyacrylamide gel and transferred to Immobilon-P membranes (Millipore). Membranes were incubated at room temperature (RT), overnight with rotation, with 100 ng of rl-domain. Western blot analysis was then performed using anti-his (Sigma, St Louis, MO), -myc [Developmental Studies Hybridoma Bank (DSHB), the University of Iowa, Iowa City (IA)], or -i-domain [Bear1 (Immunotech, Marseille, France), or H5A4 (DSHB)] antibody probes and chemiluminescent detection. Enzyme-linked immunosorbent assays (ELISA) were performed to confirm Far-Western blot analysis as outlined below.

**Enzyme-linked immunosorbent analysis for I-domain adherence**

Purified porin (200 ng) was isolated from *N. gonorrhoeae* strains UU1 (P1A) or MS11 (P1B) or MS11 pil were used to coat 96-well microtitre plates. Alternatively, microtitre plates were coated with 10² *N. gonorrhoeae* strains 1291, FA1090, or MS11 or outer membranes isolated from these same bacterial strains. Wells were rinsed and non-specific binding sites were blocked (30 min, RT) with PBS-0.25% BSA-0.05% Tween-20. One hundred microlitres of a 20 ng ml⁻¹ solution of rl-domain was added to the first of 10 wells for each gonococcal preparation tested. The concentration of I-domain was serially diluted (twofold) in each subsequent well to a final dilution of 1:512. Microtitre plates were incubated at least 2 h at 4°C after which supernatants were removed, each well was washed six times with PBS-0.05% Tween-20 and subsequently incubated (1 h, RT) in blocking buffer. ELISA were then performed according to standard protocols using anti-his, -myc, or -CD11b (H5A4 or LM2/1) primary and peroxidase-conjugated secondary antibodies. Absorbance of the o-phenylenediamine dihydrochloride peroxidase substrate was determined spectrophotometrically at 495 nm. Primary antibody was omitted from one well. A second well was coated with 10² *N. gonorrhoeae* strain MS11, FA1090, or 1291, which served as positive controls (based on Far-Western Blot analysis) for assays in which I-domain adherence to purified porin and pilin was examined.

**Acknowledgements**

We gratefully acknowledge K. A. Ault and the Department of Obstetrics and Gynecology at the University of Iowa who allow us to obtain cervical tissue biopsies used to seed the primary cell cultures used in these studies. We thank K. Ault, B. Vanderpolh and M. Ketterer for the cervical secretion specimens. Antibody H5A4 was developed by J. T. August and J. E. K. Hildreth; antibody LM2/1 was developed by T. A. Springer. These antibodies were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. This work was supported by NIH grants AI45728, AI43924, AI38515 and training grant ST32HL07638.

**References**

Alteri, D.C., and Edgington, T.S. (1988) The saturable high affinity association of factor X to ADP-stimulated monocytes defines a novel function of the Mac-1 receptor. *J Biol Chem* 263: 7007–7015.

Alteri, D.C., Wilte, W.L., and Edgington, T.S. (1990) Signal transduction initiated by extracellular nucleotides regulates the high affinity ligand recognition of the adhesion receptor CD11b/CD18. *J Immunol* 145: 662–670.

Aota, S.-I., Nagai, T., and Yamada, K.M. (1991) Characterization of regions of fibronectin besides the arginine-glycine-aspartic acid sequence required for adhesive function of the cell-binding domain using site-directed mutagenesis. *J Biol Chem* 266: 15938–15943.

Aota, S.-I., Nomizu, M., and Yamada, K.M. (1994) The short amino acid sequence pro-his-ser-arg-asn in human fibronectin enhances cell-adhesive function. *J Biol Chem* 269: 24756–24761.

Apicella, M.A. (1974) Antigenically distinct populations of Neisseria gonorrhoeae: isolation and characterization of the responsible determinants. *J Infect Dis* 130: 619–625.

Arnaout, M.A. (1990) Leukocyte adhesion molecules deficiency: its structural basis, pathophysiology and implications for modulating the inflammatory response. *Immunolog Rev* 114: 145–180.

Barnum, S.R., Ishii, Y., Agrawal, A., and Volanakis, J.E. (1992) Production and interferon-gamma-mediated regulation of complement component C2 and factors B and D by the astroglialoma cell line U105-MG. *Biochem J* 287: 595–601.

Berton, G., Laudanna, C., Sorio, C., and Rossi, F. (1992) Generation of signals activating neutrophil functions by leukocyte integrins: LFA-1 and gp150/95, but not CR3, are able to stimulate the respiratory burst of human neutrophils. *J Cell Biol* 116: 1007–1017.

Bleich, P., Planas-Basset, D., Meissner, A., and Campana, A. (1994) Investigations on the cell type responsible for the endometrial secretion of complement component 3 (C3). *Hum Reprod* 9: 1652–1659.
Bjerkes, R., Guttmersen, H.-K., Solberg, C.O., and Wetzler, L.M. (1995) Neisserial porins inhibit human neutrophil actin polymerization, degranulation, opsonin receptor expression, and phagocytosis but prime the neutrophils to increase their oxidative burst. *Infec Immun* **63**: 160–167.

Bowditch, R.D., Harirahan, M., Tominna, E.F., Smith, J.W., Yamada, K.M., Getzoff, E.D., and Ginsberg, M.H. (1991a) Identification of a novel integrin binding site in fibronectin. *J Biol Chem* **269**: 10856–10863.

Bowditch, R.D., Halloran, C.E., Aota, S.-i, Obara, M., Plow, E.F., Yamada, K.M., and Ginsberg, M.H. (1991b) Integrin $\alpha_{IIb}\beta_{3}$ (platelet GPIIb-IIIa) recognizes multiple sites in fibronectin. *J Biol Chem* **266**: 23323–23326.

Brown, E.J. (1991) Complement receptors and phagocytosis. *Curr Opin Immunol* **3**: 76–82.

Carbonetti, N.H., Simnad, V.I., Seifert, H.S., and Sparling, P.F. (1988) Genetics of protein I of *Neisseria gonorrhoeae*: construction of hybrid porins. *Proc Natl Acad Sci USA* **85**: 6841–6845.

Carbonetti, N., Simnad, V.I., Elkins, C., and Sparling, P.F. (1990) Construction of isogenic gonococci with variable porin structure: effects on susceptibility to human serum and antibiotics. * Mol Microbiol* **4**: 1009–1018.

Caron, E., and Hall, A. (1998) Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. *Science* **282**: 1717–1720.

Cohen, M.S., and Cannon, J.G. (1999) Human experimentation with *Neisseria gonorrhoeae*: progress and goals. *J Infect Dis* **179**: S375–S379.

Cohen, M.S., Cannon, J.G., Jerse, A.E., Charniga, L.M., Isbey, S.F., and Whicker, L.G. (1994) Human experimentation with *Neisseria gonorrhoeae*: rationale, methods, and implications for the biology of infection and vaccine development. *J Infect Dis* **169**: 532–537.

Cooke, S.J., Jolley, K., Ison, C.A., Young, H., and Heckels, J.E. (1998) Naturally occurring isolates of *Neisseria gonorrhoeae*, which display anomalous serovar properties, express PIA/PIB hybrid porins, deletions in PIB or novel PIA molecules. *FEBS Microbiol Lett* **162**: 75–82.

Cooper, N.R. (1991) Complement evasion strategies of microorganisms. *Immunol Today* **12**: 327–331.

Densen, P. (1989) Interaction of complement with *Neisseria meningitidis* and *Neisseria gonorrhoeae*. *Clin Microbiol Rev* **2**: S11–S17.

Densen, P. (1990) Complement. In *Principles and Practice of Infectious Disease*, 3rd edn. Mandell, G.L., Douglas, R.G. Jr., and Bennett, J.E. (eds). New York: Churchill Livingstone, pp. 62–80.

Densen, P., MacKeen, L.A., and Clark, R.A. (1982) Dissemination of gonococcal infection is associated with delayed stimulation of complement-dependent neutrophil chemotaxis in vitro. *Infec Immun* **38**: 563–572.

Dickerson, S.K., and Santoro, S.A. (1998) Ligand recognition by the I-domain-containing integrins. *CMLS Cell Mol Life Sci* **54**: 556–566.

Dillard, J. (1999) A variable pathogenicity island associated with disseminated gonococcal infection. Midwest Microbial Pathogenesis Group, Sixth Annual Midwest Microbial Pathogenesis Meeting, Milwaukee, WI.

Dovezenski, N., Billetta, R., and Gigli, I. (1992) Expression and localization of the complement system in human skin. *J Clin Invest* **90**: 2000–2012.

Dudas, K.C., and Apicella, M.A. (1988) Selection and immunochemical analysis of lipooligosaccharide mutants of *Neisseria gonorrhoeae*. *Infec Immun* **56**: 499–504.

Edwards, J.L., and Apicella, M.A. (2002) The role of lipooligosaccharide in *Neisseria gonorrhoeae* pathogenesis of cervical epithelia: lipid A serves as a C3 acceptor molecule. *Cell Microbiol* **4**: 585–598.

Edwards, J.L., Shao, J.O., Ault, K.A., and Apicella, M.A. (2000) *Neisseria gonorrhoeae* elicits membrane ruffling and cytoskeletal rearrangements upon infection of primary human endocervical and ectocervical cells. *Infec Immun* **68**: 5354–5363.

Edwards, J.L., Brown, E.J., Ault, K.A., and Apicella, M.A. (2001) The role of complement receptor 3 (CR3) in *Neisseria gonorrhoeae* infection of human cervical epithelia. *Cell Microbiol* **3**: 611–622.

Forest, K.T., and Tainer, J.A. (1997) Type-4 pilus-structure: outside to inside and top to bottom – a minireview. *Gene* **192**: 165–169.

Forest, K.T., Dunham, S.A., Koomey, M., and Tainer, J.A. (1999) Crystallographic structure reveals phosphorylated pilin from *Neisseria*: phosphoserine sites modify type IV plus surface chemistry and fiber morphology. *Mol Microbiol* **31**: 743–752.

Gasque, P., Julen, N., Ischenko, A.M., Picot, C., Mauger, C., Chauzy, C., Ripoche, J., and Fontaine, M. (1992) Expression of complement components of the alternative pathway by gioma cell lines. *J Immunol* **149**: 1381–1387.

Griffiss, J.M., O’Brien, J.P., Yamasaki, R., Williams, G.D., Rice, P.A., and Schneider, H. (1987) Physical heterogeneity of neisserial lipooligosaccharides reflects oligosaccharides that differ in apparent molecular weight, chemical composition, and antigenic expression. *Infec Immun* **55**: 1792–1800.

Haines, K.A., Yeh, L., Blake, M.S., Cristello, P., Korchak, H., and Weismann, G. (1988) Protein I, a translocatable ion channel from *Neisseria gonorrhoeae*, selectively inhibits exocytosis from human neutrophils without inhibiting O$_2$- generation. *J Biol Chem* **263**: 945–951.

Haines, K.A., Reibman, J., Tang, X., Blake, M.S., and Weismann, G. (1991) Effects of protein I of *Neisseria gonorrhoeae* on neutrophil activation: generation of diacylglycerol from phosphatidylcholine via a specific phospholipase C is associated with exocytosis. *J Cell Biol* **114**: 433–442.

Hasty, L.A., Lambris, J.D., Lessey, B.A., Pruksananonda, K., and Lyttle, C.R. (1994) Hormonal regulation of the complement components and receptors throughout the menstrual cycle. *Am J Obstet Gynecol* **170**: 168–175.

Hook, E.W., III and Handsfield, H.H. (1999) Gonococcal infections in the adult. In *Sexually Transmitted Diseases*, 3rd edn. Holmes, K.K., Mardh, P.-A., Sparling, P.F., Lemon, S.M., Stamm, W.E., Piot, P., and Wasserheit, J.N. (eds). New York: McGraw-Hill, pp. 451–466.

Hughes, P.E., and Pfaff, M. (1996) Integrin affinity modulation. *Trends Cell Biol* **8**: 359–364.

Humphries, M.J., and Newman, P. (1998) The structure of cell adhesion molecules. *Trends Cell Biol* **8**: 78–83.

Humphries, M.J., Komoriya, A., Akiyama, S.K., Olden, K., and Yamada, K.M. (1987) Identification of two distinct regions of the type III connecting segment of human plasma fibronectin that promote cell type-specific adhesion. *J Biol Chem* **262**: 6886–6892.

© 2002 Blackwell Science Ltd, *Cellular Microbiology*, 4, 571–584.
Johnston, D.M. and Cannon, J.G. (1999) Construction of mutant strains of Neisseria gonorrhoeae lacking new anti-biotic resistance markers using a two gene cassette with positive and negative selection. 
_Gene_ **236**: 179–184.

Källström, H., Islam, Md. S., Berggren, P.-O., and Jonsson, A.-B. (1998) Cell signaling by the type IV pili of pathogenic Neisseria. _J Biol Chem_ **273**: 21777–21782.

Kimizuka, F., Ohdate, Y., Kawase, Y., Shimjo, T., Taguchi, Y., Hashino, K., et al. (1991) Role of type III homology repeats in cell adhesive function within the cell-binding domain of fibronectin. _J Biol Chem_ **266**: 3034–3051.

Kishimoto, T.K., Larson, R.S., Corbi, A.L., Dustin, M.L., Staunton, D.E., and Springer, T.A. (1989) The leukocyte integrins. _Adv Immunol_ **46**: 149–182.

Kraus, D., Medof, M.E., and Mold, C. (1998) Complementary recognition of alternative pathway activators by decay-accelerating factor and factor H. _Infect Immun_ **66**: 399–405.

van der Ley, P., Heckels, J.E., Virji, M., Hoogerhout, P., and Poolman, J.T. (1991) Topology of outer membrane porins in pathogenic Neisseria spp. _Infect Immun_ **59**: 2963–2971.

Li, R., Rieu, P., Griffith, D.L., Scott, D., and Arnaout, M.A. (1998) Two functional states of the CD11b A-domain: correlations with key features of two Mr2-complexed crystal structures. _J Cell Biol_ **143**: 1523–1534.

Loftus, J.C., and Liddington, R.C. (1997) Perspectives series: cell adhesion in vascular biology. _J Clin Invest_ **99**: 2302–2306.

Lorenzen, D.R., Gunther, D., Pandit, J., Rudel, T., Brandt, E., and Meyer, T.F. (2000) Neisseria gonorrhoeae porin modifies the oxidative burst of human professional phagocytes. _Infect Immun_ **68**: 6215–6222.

McQuillen, D.P., Gulati, S., Ram, S., Turner, A.K., Jani, D.B., Heeren, T.C., and Rice, P.A. (1999) Complement processing and immunoglobulin binding to Neisseria gonorrhoeae determined in vitro simulates in vivo effects. _J Infect Dis_ **179**: 124–135.

Makino, S., van Putten, J.P., and Meyer, T.F. (1991) Phase variation of the opacity outer membrane protein controls invasion of Neisseria gonorrhoeae into human epithelial cells. _EMBO J_ **10**: 1307–1315.

Mesri, M., Pieschla, J., and Altieri, D.C. (1998) Dual regulation of ligand binding by CD11b I domain. _J Biol Chem_ **273**: 744–748.

Miyamoto, S., Akiyama, S.K. and Yamada, K.M. (1995) Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. _Science_ **267**: 883–885.

Nagai, T., Yamakawa, N., Aota, S.-I., Yamada, S.S., Akiyama, S.K., Olden, K., and Yamada, K.M. (1991) Monoclonal antibody characterization of two distinct sites required for function of the central cell-binding domain of fibronectin in cell adhesion, cell migration, and matrix assembly. _J Cell Biol_ **114**: 1295–1305.

Naughton, M.A., Botto, M., Carter, M.J., Alexander, G.J.M., Goldman, J.M., and Walport, M.J. (1996) Extrahapetic secreted complement C3 contributes to circulating C3 levels in humans. _J Immunol_ **156**: 3051–3056.

Obara, M., Kang, M.S., and Yamada, K.M. (1988) Site-directed mutagenesis of the cell-binding domain of human fibronectin: separable, synergistic sites mediate adhesive function. _Cell 53_: 649–657.

Oglesby, T.J. (1998) The complement system in reproduction. In: _Human Complement System Health Disease_. Vol.

anakis, J.E., and Frank, M.M. (eds). New York: Marcel Dekker, pp. 355–373

de la Paz, H., Cooke, S. J. and Heckels, J. E. (1995) Effect of sialylation of lipopolysaccharide of Neisseria gonorrhoeae on recognition and complement-mediated killing by monoclonal antibodies directed against different outer-membrane antigens. _Microbiol_ **141**: 913–920.

Price, R.J., and Boettcher, B. (1979) The presence of complement in human cervical mucus and its possible relevance to infertility in women with complement-dependent sperm-immobilizing antibodies. _Fertil Steril_ **32**: 61–66.

van Putten, J.P.M., Duensing, T.D., and Carlson, J. (1998) Gonococcal invasion of epithelial cells driven by PIA, a bacterial ion channel with GTP binding properties. _J Exp Med_ **188**: 941–952.

Ram, S., Sharma, A.K., Simpson, S.D., Gulati, S., McQuillen, D.P., Pangburn, M.K., and Rice, P.A. (1998a) A novel sialic acid binding site on factor H mediates serum resistance of nlsialyated Neisseria gonorrhoeae. _J Exp Med_ **187**: 743–752.

Ram, S., McQuillen, D.P., Gulati, S., Elkins, C., Pangburn, M.K., and Rice, P.A. (1998b) Binding of complement factor H to loop S of porin protein 1A: a molecular mechanism of serum resistance of nlsialyated Neisseria gonorrhoeae. _J Exp Med_ **187**: 743–752.

S112–S117.

Rice, P.A. (1989) Molecular basis for serum resistance in Neisseria gonorrhoeae. _Clin Microbiol Rev_ **2**: 5112–5117.

Rice, P.A., McCormack, W.M., and Kasper, D.L. (1986) Natural serum bactericidal activity against Neisseria gonorrhoeae isolates from disseminated, locally invasive, and uncomplicated disease. _J Infect Dis_ **124**: 2105–2109.

Rudel, T., Boxberger, H-J., and Meyer, T.F. (1996) Modulation of Neisseria porin (porB) by cytosolic ATP/GTP of target cells: parallels between pathogen accommodation and mitochondrial endosymbiosis. _Cell 85_: 391–402.

Schoolnik, G.K., Fernandez, R., Tai, J.Y., Rothbard, J., and Gotschlich, E.C. (1984) Gonococcal pili. Primary structure and receptor binding domain. _J Exp Med_ **159**: 1351–1370.

Segal, E., Bilyard, E., So, M., Storzbach, S., and Meyer, T.F. (1985) Role of chromosomal rearrangement in N. gonorrhoeae plus phase variation. _Cell 40_: 293–300.

Sparling, P.F. (1999) Biology of Neisseria gonorrhoeae. In: _Sexually Transmitted Diseases_, 3rd edn. Holmes, K.K., Mardh, P.-A., Sparling, P.F., Lemon, S.M., Stamm, W.E., Piot, P., and Waserheit, J.N. (eds). New York: McGraw-Hill, pp. 433–449.

Springall, T., Sheerin, N.S., Abe, K., Holers, V.M., Wan, H., and Sacks, S.H. (2001) Epithelial secretion of C3 promotes colonization of the upper urinary tract by Escherichia coli. _Nature Med_ **7**: 801–806.

Stephens, D.S., and Shafer, W.M. (1987) Evidence that serum resistance genetic locus sac-3 of Neisseria gonorrhoeae is involved in lipopolysaccharide structure. _J Gen Microbiol_ **133**: 2671–2679.

Swanson, K.V., Jarvis, G.A., Brooks, G.F., Barham, B.J., Cooper, M.D., and Griffiss, J.M. (2001) CEACAM is not necessary for Neisseria gonorrhoeae to adhere to and invade female genital epithelial cells. _Cell Microbiol_ 3: 681–691.

© 2002 Blackwell Science Ltd, _Cellular Microbiology_, 4, 571–584.
Symth, C.J., Marron, M.B., Twohig, J.M.G.J., and Smith, S.G.J. (1996) Fimbrial adhesins: similarities and variations in structure and biogenesis. FEMS Immunol Med Microbiol 16: 127–139.

Tobiason, D.M., and Seifert, H.S. (2001) Inverse relationship between pilus-mediated gonococcal adherence and surface expression of the pilus receptor, CD46. Microbiol 147: 2333–2340.

Vanderpuye, O.A., Labarrere, C.A., and McIntyre, J.A. (1992) The complement system in reproduction. Fertil Immunol 27: 145–155.

Vogel, U., and Frosch, M. (1999) Mechanisms of neisserial serum resistance. Mol Microbiol 32: 1133–1139.

Wetzler, L.M., Barry, K., Blake, M.S., and Gotschlich, E.C. (1992) Gonococcal lipo-oligosaccharide sialylation prevents complement-dependent killing by immune sera. Infect Immun 60: 39–43.

White, R.T., Damm, D., Hancock, N., Rosen, B.S., Lowell, B.B., Usher, P. et al. (1992) Human adipsin is identical to complement factor D and is expressed at high levels in adipose tissue. J Biol Chem 267: 9210–9213.

Wright, S.D., and Silverstein, S.C. (1983) Receptors for C3b and C3bi promote phagocytosis but not the release of toxic oxygen from human phagocytes. J Exp Med 158: 2016–2023.

Würzner, R. (1999) Evasion of pathogens by avoiding recognition or eradication by complement, in part via molecular mimicry. Mol Immunol 36: 249–260.

Xiong, J.-P., Li, R., Essafi, M., Stahle, T., and Arnaout, M.A. (2000) An isoleucine-based allosteric switch controls affinity and shape shifting in integrin CD11b A-domain. J Biol Chem 275: 38762–38767.

Yamamoto, K., and Johnston, R.B. (1984) Dissociation of phagocytosis from stimulation of the oxidative metabolic burst in macrophages. J Exp Med 159: 405–416.

Young, J.D.-E., Blake, M., Mauro, A., and Cohn, Z.A. (1983) Properties of the major outer membrane protein from Neisseria gonorrhoeae incorporated into model lipid membranes. Infect Immun 60: 39–43.

Zhang, L., and Plow, E.F. (1996) A discrete site modulates activation of I domains: application to integrin αmβ2. J Biol Chem 271: 29953–29957.