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Invasion of Human Mucosal Epithelial Cells by *Neisseria gonorrhoeae* Upregulates Expression of Intercellular Adhesion Molecule 1 (ICAM-1)†

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Infection of the mucosa by *Neisseria gonorrhoeae* involves adherence to and invasion of epithelial cells. Little is known, however, about the expression by mucosal epithelial cells of molecules that mediate cellular interactions between epithelial cells and neutrophils at the site of gonococcal infection. The aim of this study was to determine the expression of intercellular adhesion molecule 1 (ICAM-1) by epithelial cells during the process of gonococcal invasion. The highly invasive strain FA1090 and the poorly invasive strain MS11 were incubated with human endometrial adenocarcinoma (HEC-1-B) or human cervical carcinoma (ME-180) epithelial cells, after which ICAM-1 expression was measured by flow cytometry. After 15 h of infection with FA1090, expression of ICAM-1 increased 4.7- and 2.1-fold for HEC-1-B and ME-180 cells, respectively, whereas 15 h of infection of HEC-1-B cells with MS11 increased ICAM-1 expression only 1.6-fold. ICAM-1 expression was restricted to the cell surface, since no soluble ICAM-1 was detected. The distribution of staining was heterogeneous and mimicked that seen after treatment of HEC-1-B cells with the ICAM-1 agonist tumor necrosis factor alpha (TNF-α) in the absence of bacteria. PCR and dot blot analyses of ICAM-1 mRNA showed no change in levels over time in response to infection. Although TNF-α was produced by HEC-1-B cells after infection, the extent of ICAM-1 upregulation was not affected by neutralizing anti-TNF-α antiserum. Dual-fluorescence flow cytometry showed that the cells with the highest levels of ICAM-1 expression were cells with associated gonococci. We conclude that epithelial cells upregulate the expression of ICAM-1 in response to infection with invasive gonococci. On the mucosa, upregulation of ICAM-1 by infected epithelial cells may function to maintain neutrophils at the site of infection, thereby reducing further invasion of the mucosa by gonococci.

Infection of the genital mucosa by *Neisseria gonorrhoeae* involves attachment to and invasion of epithelial cells. Initial adherence of gonococci to columnar epithelial cells is mediated by type IV pili assembled from pilin subunit PilE proteins and pilus tip-associated PilC proteins (30, 31). Attachment is enhanced by the expression of phase-variable opacity-associated (Opa) proteins (19). Following internalization into epithelial cells through a process involving the polymerization of both actin microfilaments and microtubules (10, 22, 27), gonococci can be found in vacuoles and free in the cell cytoplasm (1, 33).

Despite our knowledge of the mechanisms of gonococcal invasion of epithelial cells, little is known about the immunologic consequences of gonococcal infection. One of the characteristics of acute uncomplicated gonorrhea is an intense inflammatory infiltrate consisting predominantly of neutrophils (18). Several studies demonstrate that infected epithelial cells may be the primary source of the proinflammatory and inflammatory cytokine signals for initiation of the inflammatory response to gonococcal infection. McGee et al. demonstrated that gonococcal infection of human fallopian tube mucosa resulted in increased mucosal production of tumor necrosis factor alpha (TNF-α) (20). This finding was recently expanded by Naumann et al., who reported that gonococcal infection of epithelial cells induces the upregulation of a variety of inflammatory cytokines, including TNF-α, interleukin-1β (IL-1β), IL-6, and IL-8 (23). The levels of these four cytokines are elevated in both the urine and plasma of men after intraurethral challenge with *N. gonorrhoeae* (26).

Intercellular adhesion molecule 1 (ICAM-1; CD54) is a cell surface glycoprotein that functions as a counterreceptor for the β_2-integrins lymphocyte function-associated antigen (LFA-1; CD11a/CD18) and Mac-1 (CD11b/CD18; CR3), which are expressed by neutrophils and other inflammatory cell types (2, 34). Interactions between ICAM-1 and β_2-integrins are known to mediate specific and reversible intercellular adhesion events during an inflammatory response, thereby localizing migrating neutrophils at the site of acute infection. ICAM-1 is expressed constitutively at low levels on a limited distribution of endothelial and epithelial cells (7). On cells at sites of inflammation and on cell types which do not constitutively express ICAM-1, expression can be upregulated by agonists such as the cytokines TNF-α, IL-1β, and gamma interferon (IFN-γ) (38). In addition, there is a soluble form of ICAM-1 (sICAM-1), which is most probably a form of ICAM-1 that has been split off from the membrane by proteolytic cleavage (28). The degree to which sICAM-1 interferes with the function of membrane-associated ICAM-1 remains uncertain.

The purpose of this study was to examine the expression of ICAM-1 by human mucosal epithelial cells in response to gonococcal infection. We report that despite no change in ICAM-1 mRNA levels, ICAM-1 expression was upregulated after infection with a highly invasive gonococcal strain but not

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with a poorly invasive strain. Furthermore, although gonococcal infection induced cells to produce TNF-α, upregulation of expression resulted mainly from direct contact between epithelial cells and associated gonococci. On the mucosa, upregulation of ICAM-1 expression by infected epithelial cells may function to localize neutrophils at the site of infection, thereby reducing further invasion of the mucosa by gonococci.

MATERIALS AND METHODS

***N. gonorrhoeae* strains.** Strains FA1090 (Opa^+ Pil^+; highly invasive) and MS11 (Opa^- Pil^-; poorly invasive) have been described previously (5, 8, 11, 41). Stock cultures were maintained in 10% skim milk at −70°C. The organisms were cultivated at 37°C in 5% CO_2 on gonococcal agar base (Difco, Detroit, Mich.) containing 2% IsoVitaleX (Becton Dickinson, Mountain View, Calif.). The characteristic colony morphology was used to assess the expression of Opa proteins and piliation (16, 35).

Epithelial cell lines. The human endometrial adenocarcinoma cell line HEC-1-B, the human cervical carcinoma cell line ME-180, and the human colon epithelial cell lines HT29 and Caco-2 were obtained from the American Type Culture Collection (Rockville, Md.) and have been characterized previously (9, 29, 36, 40). The use of HEC-1-B and ME-180 cells for the study of gonococcal infection of epithelial cells has been described previously (13, 22, 23, 33). HEC-1-B cells were cultured in Eagle minimal essential medium with Earle's balanced salt solution containing 10% (vol/vol) fetal bovine serum (HyClone, Logan, Utah), 1% nonessential amino acids, and 1× sodium pyruvate. ME-180 and HT29 cells were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum. The medium for HT29 cells also contained 100 U of penicillin per ml and 100 μg of streptomycin per ml. Caco-2 cells were cultured in Eagle minimal essential medium with Earle's balanced salt solution containing 20% fetal bovine serum, 1% nonessential amino acids, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. All media and supplements except those indicated were indicated from the Cell Culture Facility, University of California at San Francisco.

Bacterial infection of epithelial cells. For bacterial infection assays, 10^6 HEC-1-B or ME-180 cells were seeded into wells of a six-well cell culture plate 24 h before introduction of the bacteria. At this seeding density, the cells were approximately 80% confluent at the time of the experiment. Bacteria grown overnight on plates were suspended in GC medium (Difco), washed twice, and resuspended to a concentration of 10^9 bacteria per ml based on the optical density at 650 nm. Aliquots of 100 μl of bacteria were added to the culture plate wells containing the cells, and the monolayers were incubated at 37°C in 5% CO_2. At specified periods, cells were harvested from the wells, washed twice, and tested for expression of ICAM-1 by flow cytometry. For some experiments, the immunoglobulin G (IgG) fraction of rabbit polyclonal anti-human TNF-α antiserum (Calbiochem, San Diego, Calif.), which was a neutralizing antiserum, was added at 20 μg/ml to the cell monolayers prior to bacterial infection, and in control wells the cells were treated with 20 μg of normal rabbit IgG (Biodesign International, Kennebunk, Maine) per ml.

In the dual-label fluorescence experiments, 10^9 gonococci in GC medium were labeled with CellTracker Orange CMTMR (Molecular Probes, Eugene, Oreg.) at a final concentration of 5 μM for 45 min at 37°C. The bacteria were washed twice and incubated in GC medium without the fluorescent dye for 30 min. Aliquots of 100 μl of the labeled bacteria were then added to 10^6 HEC-1-B cells as described above.

For some experiments, 10^6 cells of either HEC-1-B, HT29, or Caco-2 cell lines were stimulated for 15 h in the absence of bacteria with TNF-α (Sigma, St. Louis, Mo.) at a final concentration of either 100 ng/ml or as specified, after which the cells were washed twice and expression of ICAM-1 was determined by flow cytometry.

Flow cytometry. For single- and dual-label flow cytometric analysis of ICAM-1 expression, 10^6 cells were incubated for 30 min on ice with 2 μg of a monoclonal antibody against CD54 (Chemicon International, Temecula, Calif.) in a total volume of 100 μl of phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). After two washes with PBS-BSA, the cells were incubated for 30 min on ice with 100 μl of a 1:50 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma). The cells were washed twice with PBS-BSA and then fixed with 1% paraformaldehyde. In controls, the cells were incubated with an irrelevant mouse IgG1 isotype (Organon Teknika, Durham, N.C.). Immunofluorescence of either the FITC single label or the FITC and CellTracker Orange dual label was measured with a Becton Dickinson FACScan flow cytometer equipped with Lysis II software for data acquisition and analysis.

RNA extraction and dot blot analysis. Total cellular RNA was isolated by using an acid guanidinium thiocyanate-phenol-chloroform (TriPure reagent) method as described by the manufacturer (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). RNA (15 μg) was dotted onto Hybond N^+ (Amersham Life Science, Arlington Heights, Ill.) and fixed to the membrane by soaking for 5 min in 50 mM NaOH followed by 10 min in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Prior to hybridization, the blots were treated at 42°C for 2 h in 1× prehybridization solution (Gibco BRL, Gaithersburg, Md.) containing 50% formamide. Hybridizations were carried out at 65°C overnight by adding 10^5 to 10^6 cpm of ^{32}P-labeled ICAM-1 cDNA probe to the prehybridization solution. The membranes were washed twice at room temperature for 15 min in 2× SSC and twice again at 65°C in 0.2× SSC in the presence and absence of 25% formamide and then exposed to X-ray film at −70°C. Subsequently, the blots were stripped of their probes by two or three washes at 65°C for 1 h in 50% formamide containing 1% sodium dodecyl sulfate and rehybridized with a ^{32}P-labeled cDNA fragment of 18S rRNA. The relative intensity of each blot hybridization dot was determined by scanning densitometry, after which the intensities were normalized by calculating the ratio of the dot intensity to that of the corresponding rehybridized 18S rRNA dot. Each normalized ratio was calculated as a percentage of the maximal ratio within the experimental assay, and the significance of the intensity differences was assessed by Student's *t* test.

^{32}P-labeled cDNA probes were prepared by PCR. The template for the ICAM-1 probe was *Sal*I-digested pCD1.8, which contains the complete coding sequence of human ICAM-1 as a 1.8-kb fragment subcloned into the expression vector CDM8 (34) (kindly provided by T. Springer, Center for Blood Research, Boston, Mass.). The template for 18S rRNA was a PCR product that was amplified from HEC-1-B total RNA by the primers 5′ CTGTGATGCCCTTA GATGTCCG (forward) and 5′ ATGACCCGCACTTACTGGGAAT (reverse). The final reaction conditions for probe production were 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ (ICAM-1) or 2 mM $MgCl_2$ (18S rRNA), 2 μM reverse primer (ICAM-1, 5′ CTCTGGCTTCGTCAGAATCAC), 1 μM [^{32}P]dCTP (3000 Ci/mmol), 100 μM (each) deoxynucleoside triphosphates (dNTPs) minus dCTP, and 2.5 U of Amplitaq Gold (Perkin-Elmer, Alameda, Calif.). The reaction mixtures were heated to 95°C for 10 min and then cycled 45 times at 95°C for 50 s, 55°C for 50 s, and 72.5°C for 1.5 min and given a final elongation at 72.5°C for 10 min. PCR products were purified over QIA-quick-spin columns (Qiagen, La Jolla, Calif.).

RT-PCR. Reverse transcription reactions were carried out in 20-μl volumes that contained 5 μg of total RNA purified as described above, 1× PCR buffer (10 mM Tris-HCl [pH 9.0], 50 mM KCl, 2.5 mM $MgCl_2$), 0.5 mM dNTPs, and 0.5 μg of oligo(dT) (Promega, Madison, Wis.). The mixtures were heated to 42°C for 1 min, and then 200 U of Superscript II (Gibco BRL, Gaithersburg, Md.) reverse transcriptase was added. The mixtures were incubated at 42°C for 50 min and then heated to 75°C for 10 min.

Duplex PCRs were performed by a modification of the method of Wong et al. (42). Each reaction mixture contained 5 μl of the RT reaction mixture, 1× PCR buffer, 100 μM dNTPs, 0.33 μM [^{32}P]dCTP (3,000 Ci/mmol), 25 pmol of each forward and reverse primer, and 4 U of Amplitaq Gold in a total volume of 25 μl. A 400-bp ICAM-1 product was amplified by the primers 5′ AGTCACCTA TGGCAACGACTCC (forward) and 5′ GGCCATACAGGACACGAAGCT (reverse). A 180-bp β-actin product was amplified by the primers 5′ CAAAGTTCAC AATGTGGCCGA (forward) and 5′ GCAATGCTATCACCTCCCCTG (reverse). The reaction mixtures were heated to 95°C for 5 min and then cycled 10 times at 95°C for 50 s, 55°C for 50 s, and 72.5°C for 1.5 min and given a final elongation at 72.5°C for 10 min. Ten cycles were found to be within the exponential phase and not the saturation phase of the amplification curve for each product (results not shown). PCR products were purified over QIA-quick-spin columns. Aliquots of 2 to 6 μl were separated through 5% polyacrylamide gels, dried, and exposed to X-ray film at −70°C. The relative intensity of each RT-PCR band was determined by scanning densitometry, after which the intensity values were normalized by calculating the ratio of the ICAM-1 band to that of the corresponding β-actin control band. Each normalized ratio was calculated as a percentage of the maximal ratio within the experimental assay, and the significance of the intensity differences was assessed by Student's *t* test.

sICAM-1 and cytokine ELISAs. HEC-1-B cells (10^6) were exposed to 10^8 strain FA1090 bacteria for 1, 2, 4, or 15 h as described above. At each time point, aliquots of cell culture supernatant were removed and assayed for the presence of sICAM-1 by an enzyme-linked immunosorbent assay specific for sICAM-1 (Biotrak ELISA) as described by the manufacturer (Amersham). The assay was also repeated after concentration of the cell culture supernatant 10-fold with Centricon microconcentrators (Amicon, Beverly, Mass.). The sensitivity of the assay for sICAM-1 was <3.4 ng/ml. For cytokine determinations, aliquots of cell culture supernatant were removed 15 h after adding the bacteria and assayed for the presence of TNF-α and IL-1β by a commercial ELISA (TiterScreen I EIA; PerSeptive Diagnostics, Cambridge, Mass.). The detection limit of the assay was 28.1 pg/ml for TNF-α and 2.69 pg/ml for IL-1β.

RESULTS

ICAM-1 and sICAM-1 expression by epithelial cells. We investigated the expression of the adhesion molecule ICAM-1 on mucosal epithelial cells during the process of gonococcal adherence and invasion by using the human endometrial adenocarcinoma cell line HEC-1-B as a model system for these events. Monolayers of HEC-1-B cells were cocultured with gonococcal strains FA1090 and MS11 for 15 h, and the quantitative expression of ICAM-1 was determined by flow cytom-

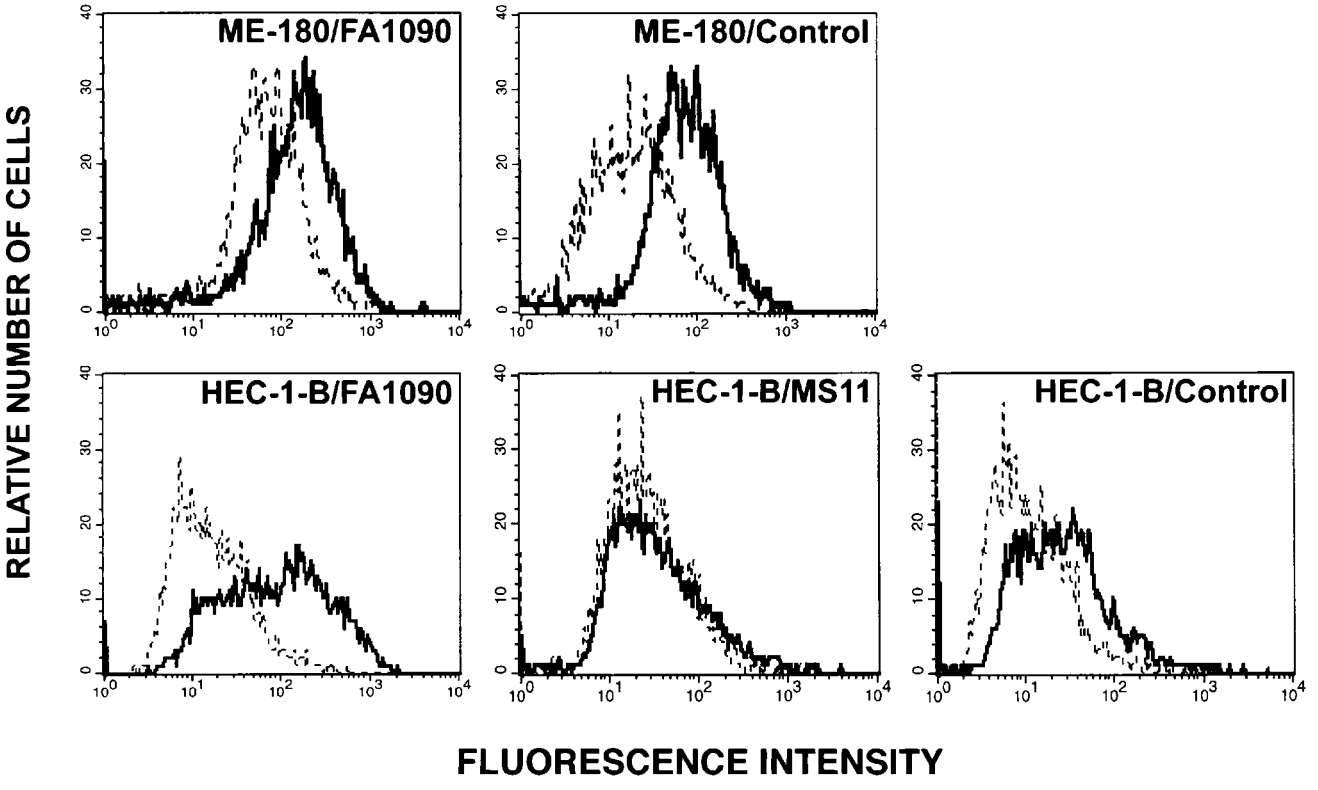

FIG. 1. Flow cytometric analysis of the expression of ICAM-1 by HEC-1-B and ME-180 cells following infection for 15 h with gonococcal strains FA1090 and MS11. For the panels showing cells infected with FA1090 and MS11, the solid line represents ICAM-1 staining after gonococcal infection and the dashed line represents ICAM-1 staining in the absence of gonococci. For the control panels, the solid line represents ICAM-1 staining in the absence of gonococci and the dashed line represents staining with an irrelevant mouse IgG_1 as the primary antibody. Data are from a representative experiment. Similar results were obtained in at least three independent experiments.

etry. As shown in Fig. 1, infection of HEC-1-B cells with the highly invasive strain FA1090 markedly upregulated the expression of ICAM-1. In contrast, exposure of HEC-1-B cells to the poorly invasive strain MS11 did not upregulate ICAM-1 expression. In the absence of bacteria, HEC-1-B cells were found to constitutively express low levels of ICAM-1. We repeated the infectivity assay with strain FA1090 and human cervical carcinoma cell line ME-180 to determine whether ICAM-1 upregulation was inducible in another mucosal epithelial cell type in response to gonococcal infection. As shown in Fig. 1, infection of ME-180 cells with FA1090 increased ICAM-1 expression 2.1-fold, although in the absence of bacteria, ME-180 cells constitutively expressed 2.0-fold more ICAM-1 than HEC-1-B cells did.

A time course analysis of the expression of ICAM-1 by HEC-1-B cells following infection by strains FA1090 and MS11 is shown in Fig. 2. The strains were incubated with HEC-1-B cells for increasing periods, after which ICAM-1 expression was measured by flow cytometry. With strain FA1090, expression of ICAM-1 increased 1.3-fold at 2 h and 2.2-fold at 4 h and continued to increase to 4.7-fold over constitutive levels at 15 h. In contrast, strain MS11 increased ICAM-1 expression only 1.6-fold at 15 h.

In addition, increased membrane expression of ICAM-1 by HEC-1-B cells after exposure to strain FA1090 bacteria did not correlate with increased or even detectable levels of sICAM-1 in cell culture supernatants. Incubation of HEC-1-B cells with strain FA1090 for 1, 2, 4, and 15 h resulted in concentrations of sICAM-1 in the supernatant and 10-fold-concentrated supernatant which did not exceed the lower limit of sensitivity of the ELISA (3.4 ng/ml) for any of the time points.

TNF-α stimulation of ICAM-1 expression. Although the distribution of ICAM-1 expression by HEC-1-B cells following infection with strain FA1090 was unimodal as judged by flow cytometry, the distribution was also broad, suggesting a heterogeneous pattern of upregulation of ICAM-1 by the cells within the population. To determine whether the broad distribution of ICAM-1 upregulation was unique to the interaction of HEC-1-B cells with gonococci or unique to HEC-1-B cells per se independent of their interaction with gonococci, we treated HEC-1-B cells with the ICAM-1 agonist TNF-α in the absence of bacteria and quantitated ICAM-1 expression by flow cytometry. Human colon epithelial cell lines HT29 and Caco-2, which are known to upregulate ICAM-1 either strongly (HT29) or weakly (Caco-2) in response to TNF-α stimulation (12), were run as controls along with the HEC-1-B cells. Figure 3 shows that HEC-1-B and HT29 cells significantly upregulated ICAM-1 expression in response to treatment with TNF-α whereas the level of expression by Caco-2 cells remained unchanged. Interestingly, the distribution of ICAM-1 expression by HEC-1-B cells after treatment with TNF-α was broader than that of HT29 cells and, more importantly, mimicked the broad distribution seen after stimulation of HEC-1-B cells with strain FA1090 gonococci. This suggests that HEC-1-B cells are not uniform in their potential to upregulate cell surface ICAM-1 expression in response to either bacterial infection or cytokine stimulation.

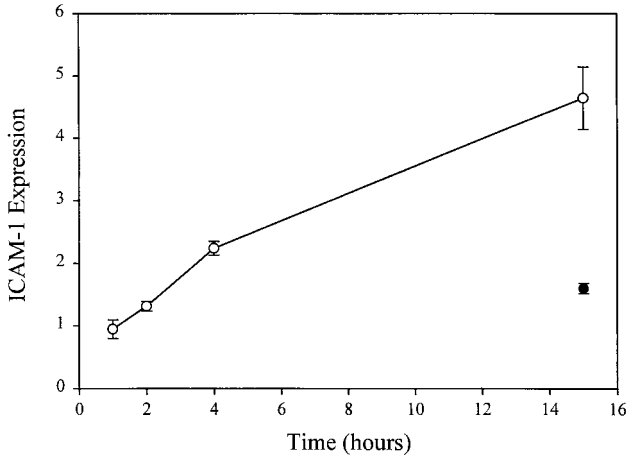

FIG. 2. Time course analysis of the expression of ICAM-1 by HEC-1-B cells following infection with gonococcal strains FA1090 and MS11. ICAM-1 staining was measured by flow cytometry, and the expression of ICAM-1 was calculated as the ratio of the mean fluorescence intensity of HEC-1-B cells in the presence of gonococci to that of HEC-1-B cells in the absence of gonococci. Values represent the mean ± standard deviation of triplicate determinations and are representative of two independent experiments. Open circles, FA1090; solid circle, MS11.

ICAM-1 mRNA levels. To further characterize the mechanism by which epithelial cells upregulate ICAM-1 expression in response to gonococcal invasion, levels of ICAM-1 mRNA were determined by RT-PCR and dot blot analyses. As shown in Fig. 4, cocultivation of HEC-1-B cells with strain FA1090 bacteria for 1, 4, and 15 h did not change the ICAM-1 mRNA levels compared with constitutive mRNA expression levels ($P > 0.05$ for all time points in both assay systems). This indicates that increased ICAM-1 expression after gonococcal invasion is the result of translational or posttranslational regulation of the level of expressed ICAM-1.

Cytokine secretion and ICAM-1 expression. Since we found that TNF-α upregulated the expression of ICAM-1 by HEC-1-B cells in the absence of bacteria, we next investigated whether gonococcal invasion of HEC-1-B cells and the resultant upregulation of ICAM-1 expression was due to the secretion of TNF-α or an additional ICAM-1 agonist cytokine, IL-1β (38). HEC-1-B cells were infected with strain FA1090 gonococci for 15 h, after which cell culture supernatants were assayed for TNF-α and IL-1β. As determined by ELISA, the concentration of TNF-α in the supernatants was 255.4 ± 35.0 pg/ml and that of IL-1β was 14.2 ± 0.8 pg/ml. Neither cytokine was detectable in HEC-1-B cell culture supernatants in the absence of gonococcal infection.

This result showed that significant amounts of TNF-α but not IL-1β were released by HEC-1-B cells following gonococcal infection, but it also raised the question whether such a concentration of TNF-α was sufficient to upregulate ICAM-1 expression after gonococcal infection to the extent observed. To answer this question, HEC-1-B cells were treated with increasing concentrations of TNF-α in the absence of gonococci for 15 h, after which ICAM-1 expression was determined by flow cytometry. As can be seen in Fig. 5, 250 pg of TNF-α per ml increased ICAM-1 expression only 1.4-fold and 10 ng of TNF-α per ml, which was a 40-fold-greater concentration of TNF-α than that detected in the cell culture supernatant, increased ICAM-1 expression only 2.1-fold compared with the 4.7-fold increase in ICAM-1 expression after gonococcal infection.

In an alternative approach to determine the importance of TNF-α in ICAM-1 upregulation by HEC-1-B cells following gonococcal infection, we added neutralizing anti-TNF-α antibody to the cell cultures prior to gonococcal infection, so that the antibody was present throughout the subsequent 15-h incubation period with the gonococci. As shown in Fig. 6, the extent of ICAM-1 upregulation was not affected by anti-TNF-α antisera. The degree to which ICAM-1 was upregulated following gonococcal infection was the same for treatments with either anti-TNF-α antiserum or normal rabbit IgG as the upregulation in untreated cells ($P > 0.05$ for either treatment). Taken together, these data indicate that TNF-α secreted by HEC-1-B cells in response to gonococcal infection played a minor role at most in the upregulation of ICAM-1 expression.

ICAM-1 upregulation and colocalization of gonococci. The finding that TNF-α secretion contributed minimally to ICAM-1

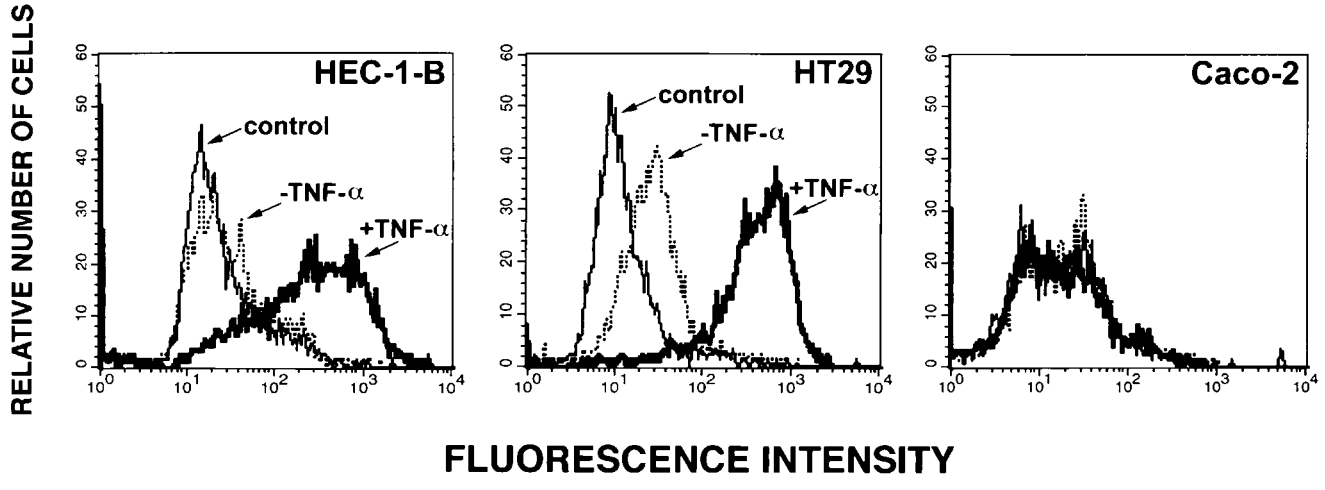

FIG. 3. Flow cytometric analysis of the expression of ICAM-1 by HEC-1-B, HT29, and Caco-2 cells after stimulation with TNF-α. Cells were either untreated or treated with 100 ng of TNF-α per ml for 15 h, after which they were stained for ICAM-1 expression. For the control histograms, the cells were treated with TNF-α and stained with an irrelevant mouse IgG1 as the primary antibody. The histograms representative of the expression of ICAM-1 by Caco-2 cells superimpose each other and are therefore not individually identified. Data are from a representative experiment. Similar results were obtained in two independent experiments.

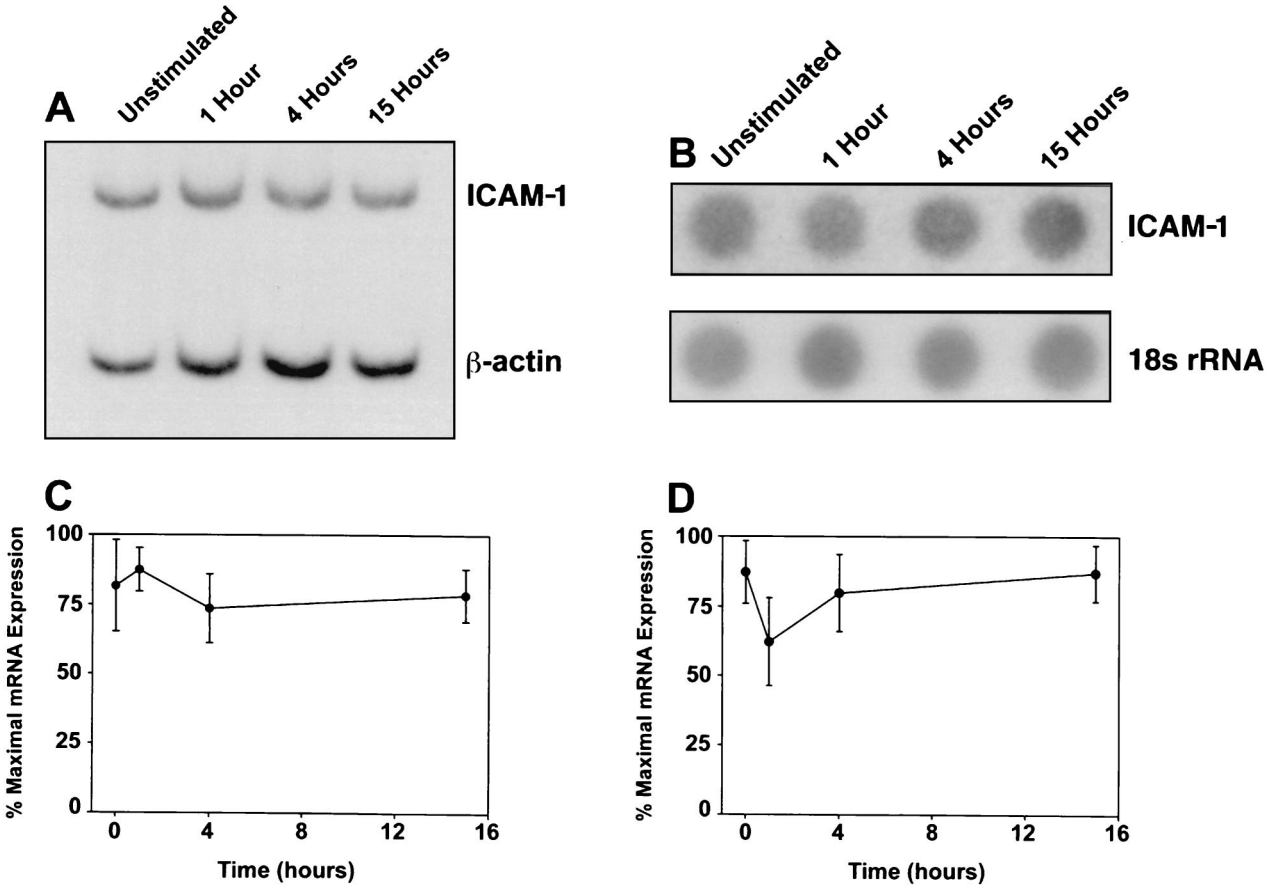

FIG. 4. RT-PCR and dot blot analyses of ICAM-1 mRNA expression by HEC-1-B cells following infection with gonococcal strain FA1090. Cells were incubated with gonococci for the indicated periods, after which total cellular RNA was extracted and analyzed. (A) RT-PCR analysis of ICAM-1 and β-actin mRNA expression. Results are from a representative experiment. (B) Dot blot hybridization analysis of ICAM-1 mRNA and 18S rRNA expression. Results are from a representative experiment. (C and D) The relative intensity of each RT-PCR band (C) or each blot hybridization dot (D) was determined by scanning densitometry, after which the intensity values were normalized by calculating the ratio of the ICAM-1 band or dot intensity to that of the corresponding control band (β-actin) or rehybridized dot (18S rRNA). Each normalized ratio was calculated as a percentage of the maximal ratio within the experimental assay. Values represent the mean ± standard deviation of triplicate determinations and are representative of three independent experiments.

upregulation following gonococcal invasion led us to investigate whether the level of ICAM-1 expression correlated with a direct interaction between gonococci and HEC-1-B cells. Fluorescence-labeled gonococci were incubated with HEC-1-B cells, and the degree of colocalization between gonococci and ICAM-1 upregulation was determined by two-color flow cytometry. As shown in Fig. 7A and C, ICAM-1 expression by HEC-1-B cells was upregulated following gonococcal infection, which is consistent with the results in Fig. 1. As can be seen in Fig. 7A, the majority of HEC-1-B cells with the highest relative fluorescence intensity for ICAM-1 expression following gonococcal infection also stained with the highest relative fluorescence intensity for gonococci. Of the HEC-1-B cells that stained with a relative fluorescence intensity greater than 10 (Fig. 7A, upper and lower right quadrants), 26.4% of a total of 30.9% were associated with the most intense gonococcal fluorescence, suggesting that the HEC-1-B cells which expressed the most ICAM-1 had the greatest number of gonococci associated with them. As a control, Fig. 7B shows the relative fluorescence intensity of staining of HEC-1-B cells for ICAM-1 following gonococcal infection by using an irrelevant primary antibody.

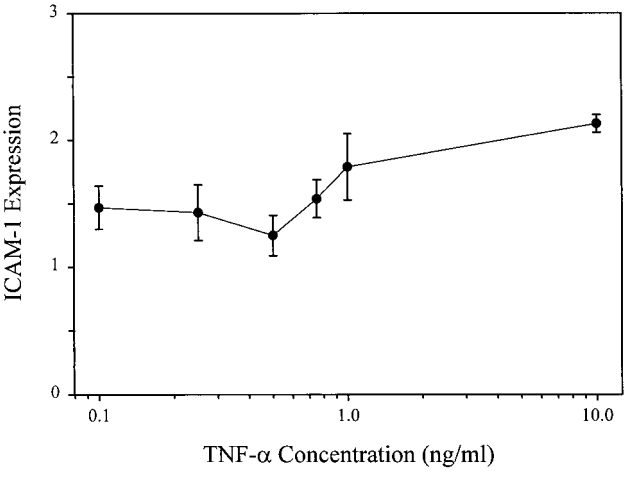

FIG. 5. Dose-response analysis of the effect of TNF-α on the expression of ICAM-1 by HEC-1-B cells. Cells were treated with the indicated concentrations of TNF-α for 15 h, after which they were stained for ICAM-1 and analyzed by flow cytometry. ICAM-1 expression was calculated as the ratio of the mean fluorescence intensity of cells treated with TNF-α to that of untreated cells. Values represent the mean ± standard deviation of triplicate determinations and are representative of two independent experiments.

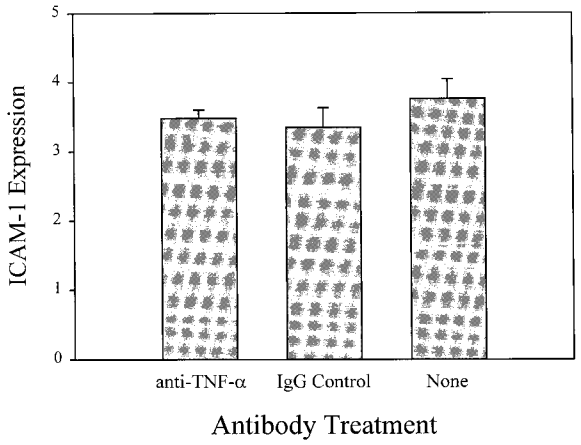

FIG. 6. Effect of neutralizing anti-TNF-α antiserum on ICAM-1 upregulation following gonococcal infection. HEC-1-B cells were incubated without (control) or with 20 μg of either the IgG fraction of rabbit polyclonal anti-human TNF-α antiserum or normal rabbit IgG per ml prior to gonococcal infection. Antibody was present throughout the subsequent 15-h incubation period with the gonococci. ICAM-1 staining was measured by flow cytometry, and the expression of ICAM-1 was calculated as the ratio of the mean fluorescence intensity of HEC-1-B cells in the presence of gonococci to that of HEC-1-B cells in the absence of gonococci. Values represent the mean ± standard deviation of triplicate determinations and are representative of two independent experiments.

DISCUSSION

Acute uncomplicated gonorrhea is characterized by an intense inflammatory infiltrate consisting predominantly of neutrophils. Little is known, however, about the expression by mucosal epithelial cells of molecules that mediate cellular interactions between epithelial cells and neutrophils at the site of gonococcal infection. In this report, we demonstrate that epithelial cells upregulate the expression of membrane ICAM-1 but not sICAM-1 in response to gonococcal infection. Detectable upregulation occurred within 2 to 4 h after infection, and cells expressing the highest levels of ICAM-1 had the largest number of gonococci associated with them. Although the extent of upregulation on ME-180 cells after infection was approximately 50% of that seen with HEC-1-B cells, the constitutive expression of ICAM-1 on ME-180 cells in the absence of bacteria was twice that on HEC-1-B cells. Taken together, these results indicate that mucosal epithelial cells can function to localize extravasating neutrophils not only to the site of gonococcal infection but, more specifically, to those epithelial cells with the highest multiplicity of infection. Adherence of neutrophils to highly infected epithelial cells may also contribute to the destruction or shedding of epithelial cells, as seen in urethral exudates from men with gonococcal urethritis (1).

Cytokines are important regulators of ICAM-1 expression, with TNF-α and IL-1β being recognized as relatively universal ICAM-1 agonists (38). We tested TNF-α as an ICAM-1 agonist for HEC-1-B cells and found that it upregulates the expression of ICAM-1 by these cells. Furthermore, ELISA analyses of HEC-1-B cell culture supernatants following gonococcal infection revealed the presence of significant concentrations of TNF-α but not of IL-1β. However, TNF-α dose-response experiments showed that the amount of TNF-α produced was not sufficient to upregulate ICAM-1 expression after gonococcal infection to the extent observed. Furthermore, infection experiments done with neutralizing anti-TNF-α antiserum showed no difference in the degree of ICAM-1 upregulation in cells incubated with gonococci in the presence of the antiserum compared with that in untreated infected cells. These data suggest that TNF-α is at most only partially responsible for ICAM-1 upregulation following gonococcal infection of HEC-1-B cells and therefore that a second mechanism, involving the direct interaction of HEC-1-B cells and gonococci, may exist for ICAM-1 upregulation.

The pattern of upregulation of ICAM-1 after either gonococcal infection or TNF-α stimulation was heterogeneous for HEC-1-B cells, suggesting that they are not uniform in their potential to upregulate ICAM-1. This may reflect a difference in the relative differentiation state of the cells within the population. Since HEC-1-B cells can be induced by laminin to express a more differentiated phenotype in vitro (3), it may be that the cells in a population are not uniformly dedifferentiated, which in turn may influence ICAM-1 expression. This

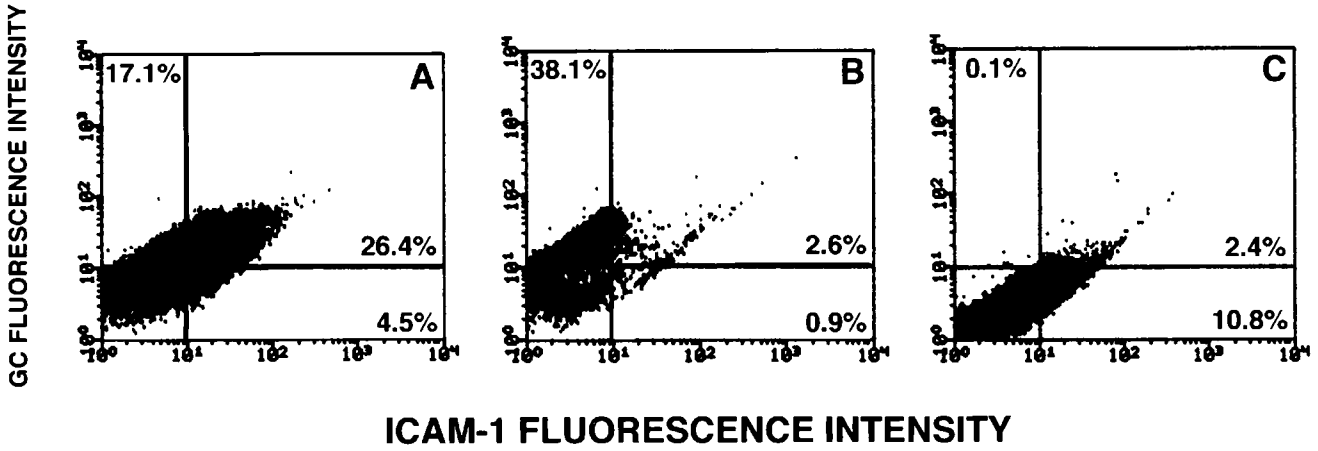

FIG. 7. Two-color flow cytometric analysis of ICAM-1 expression by HEC-1-B cells in relation to the colocalization of gonococci with HEC-1-B cells. Fluorescence-labeled gonococci were incubated with HEC-1-B cells for 15 h, after which the cells were stained for ICAM-1 expression. The relative fluorescence intensities of both fluorescent labels were measured by flow cytometry. (A) HEC-1-B cells incubated with fluorescence-labeled gonococci and then stained for ICAM-1; (B) HEC-1-B cells incubated with fluorescence-labeled gonococci and then stained with an irrelevant mouse IgG1 as the primary antibody; (C) HEC-1-B cells stained for ICAM-1 in the absence of bacteria. The percentage in each quadrant of the panels represents the percentage of HEC-1-B cells within the entire population in that given quadrant. Data are representative of two independent experiments.

interpretation is supported by the finding that less differentiated keratinocytes in culture express more ICAM-1 than do differentiated cells after stimulation by ICAM-1 agonist IFN-γ (15). Furthermore, the differentiation state of a cell has been reported to determine the susceptibility of the cell to bacterial invasion (4). Although both *Salmonella typhimurium* and *Listeria monocytogenes* invade Caco-2 cells, Coconnier et al. found that the former bacteria invade differentiated cells whereas the latter invade undifferentiated cells (4). It is possible that the differentiation state of an individual HEC-1-B cell also influences its susceptibility to gonococcal infection. If so, differences in the relative differentiation state between HEC-1-B cells may account for our finding that HEC-1-B cells which expressed the most ICAM-1 after gonococcal infection had the largest number of cell-associated gonococci.

Several studies have reported that inflammatory cytokines are produced by epithelial cells in response to gonococcal infection. McGee et al. demonstrated that gonococcal infection of human fallopian tube mucosa resulted in increased production of TNF-α (20). A recent study by Naumann et al. showed that gonococcal infection of three different epithelial cell lines induced the upregulation of a variety of proinflammatory and inflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-8 but not including IFN-γ (23). They found that prior to cytokine induction, the transcription factor nuclear factor κB was activated within 10 min after infection followed by cytokine mRNA induction at 15 min, with synthesis and release of the cytokines weakly until 3 h postinfection. The same four cytokines have elevated levels in both the urine and plasma of men after experimental challenge with *N. gonorrhoeae* (26). The relatively early time course of expression of TNF-α, IL-6, and IL-8 versus the late expression of IL-1β led the authors to suggest that TNF-α, IL-6, and IL-8 were produced by the urethral epithelium at the site of local infection whereas IL-1β was derived from infiltrating neutrophils. Our data indicate that TNF-α produced by mucosal epithelial cells in response to local infection may function in part to upregulate ICAM-1 expression by surrounding epithelial cells. If IL-1β is produced by neutrophils transmigrating into the area of infection, upregulation of ICAM-1 expression by epithelial cells may be amplified.

Gonococcal infection of HEC-1-B cells had no upregulatory effect on levels of ICAM-1 mRNA over time, despite significant increases in ICAM-1 protein expression on cell membranes. This indicates that increased expression of ICAM-1 by HEC-1-B cells following gonococcal infection is mediated at the translational and/or posttranslational level. Several mechanistic possibilities underlie this suggestion: (i) an increase in posttranslational processing and maturation of ICAM-1; (ii) an increase in ICAM-1 mRNA translation; and (iii) an increase in the half-life of the intracellular pool of ICAM-1 bound for membrane expression. The report that reactive oxygen species result in posttranslational modifications of ICAM-1 supports the concept of a posttranslational interpretation of the data (32). Interestingly, our data contrasts with the demonstration that invasive enteric bacteria upregulate both ICAM-1 protein and mRNA expression by intestinal epithelial cells (12). This suggests the existence of different mechanisms of ICAM-1 upregulation in response to bacterial invasion, depending on the type of epithelial cell as well as the phenotypic characteristics of the infecting organism.

Gonococci have been shown both in vitro and in vivo to activate complement, resulting in the cell surface deposition predominantly of iC3b fragments of complement component C3 (14, 21). Because both cervical mucus and seminal plasma contain a fully functional complement cascade (25, 37), it is likely that gonococci and their elaborated outer membrane blebs activate complement during the course of most natural gonococcal infections (24). Activation of the complement cascade can enhance ICAM-1 upregulation induced by TNF-α. Vaporciyan et al. found that TNF-α-dependent upregulation of lung vascular ICAM-1 in vivo required the availability of complement (39), and Kilgore et al. demonstrated that the complement membrane attack complex enhanced TNF-α-induced endothelial cell expression of ICAM-1 (17). It will be interesting to determine whether complement activation by gonococci can enhance ICAM-1 upregulation induced by either TNF-α or the direct interaction of gonococci and epithelial cells, as we report herein. In addition, iC3b deposited on intact gonococci and on gonococcal outer membrane blebs may function as a ligand for Mac-1 on neutrophils (6). However, as a result of their interaction with Mac-1, iC3b ligands may also block the binding of neutrophil Mac-1 to epithelial-cell ICAM-1, thereby inhibiting the recognition of the inflammatory site of infection by transmigrating neutrophils.

In conclusion, our results demonstrate that gonococcal infection of mucosal epithelial cells resulted in the upregulation of ICAM-1 expression. Although TNF-α was produced by HEC-1-B cells following gonococcal infection and functioned as an ICAM-1 agonist for HEC-1-B cells when added exogenously to cultured cells, the quantity of TNF-α produced after infection did not account for the extent to which ICAM-1 was upregulated. In addition, neutralizing anti-TNF-α antiserum did not affect the level of ICAM-1 upregulation as a result of infection. A second mechanism of ICAM-1 upregulation involving the direct interaction of gonococci with HEC-1-B cells requires further investigation. The synthesis by infected epithelial cells of cytokines that chemoattract and activate neutrophils (20, 23, 26) followed by the upregulation of epithelial cell ICAM-1 expression to maintain neutrophils at the site of infection provides evidence of an important role for mucosal epithelial cells in the inflammatory process in response to mucosal gonococcal infection.

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REFERENCES

1. **Apicella, M. A., M. Ketterer, F. K. Lee, D. Zhou, P. A. Rice, and M. S. Blake.** 1996. The pathogenesis of gonococcal urethritis in men: confocal and immunoelectron microscopic analysis of urethral exudates from men infected with *Neisseria gonorrhoeae*. J. Infect. Dis. **173:**636–646.
2. **Arnaout, M. A.** 1990. Structure and function of the leukocyte adhesion molecules CD11/CD18. Blood **75:**1037–1050.
3. **Behrens, P., C. Meissner, H. Hopfer, J. Scheumann, M. I. Tan, N. Ellerbrake, E. Strunck, and G. Vollmer.** 1996. Laminin mediates basement membrane induced differentiation of HEC 1B endometrial adenocarcinoma cells. Biochem. Cell Biol. **74:**875–886.
4. **Coconnier, M. H., M. F. Bernet, S. Kerneis, G. Chauviere, J. Fourniat, and A. L. Servin.** 1993. Inhibition of adhesion of enteroinvasive pathogens to human intestinal Caco-2 cells by *Lactobacillus acidophilus* strain LB decreases bacterial invasion. FEMS Microbiol. Lett. **110:**299–305.
5. **Cohen, M. S., J. G. Cannon, A. E. Jerse, L. M. Charniga, S. F. Isbey, and L. G. Whicker.** 1994. Human experimentation with *Neisseria gonorrhoeae*: rationale, methods, and implications for the biology of infection and vaccine development. J. Infect. Dis. **169:**532–537.
6. **Dierich, M. P., T. F. Schulz, A. Eigentler, H. Huemer, and W. Schwable.** 1988. Structural and functional relationships among receptors and regulators of the complement system. Mol. Immunol. **25:**1043–1051.
7. **Dustin, M. L., R. Rothlein, A. K. Bhan, C. A. Dinarello, and T. A. Springer.** 1986. Induction by IL 1 and interferon-γ: tissue distribution, biochemistry, and function of a natural adherence molecule ICAM-1. J. Immunol. **137:** 245–254.

8. **Edwards, M., R. L. McDade, G. Schoolnik, J. B. Rothbard, and E. C. Gotschlich.** 1984. Antigenic analysis of gonococcal pili using monoclonal antibodies. J. Exp. Med. **160:**1782–1791.
9. **Forsman, L. M.** 1987. An apocrine membrane antigen with polarized distribution and hormonally regulated expression in human endometrial and mammary carcinoma cell lines. Acta Pathol. Microbiol. Immunol. Scand. **95:**315–323.
10. **Grassme, H. U., R. M. Ireland, and J. P. van Putten.** 1996. Gonococcal opacity protein promotes bacterial entry-associated rearrangements of the epithelial cell actin cytoskeleton. Infect. Immun. **64:**1621–1630.
11. **Griffiss, J. M., C. J. Lammel, S. Zhao, J. Wang, N. P. Dekker, and G. F. Brooks.** 1994. Pili and Opa are required for HEC-1-B cell microvillus elongation and engulfment of *Neisseria gonorrhoeae*, p. 76–77. *In* J. S. Evans, S. E. Yost, M. C. J. Maiden, and I. M. Feavers (ed.), Proceedings of the IX International Pathogenic *Neisseria* Conference.
12. **Huang, G. T., L. Eckmann, T. C. Savidge, and M. F. Kagnoff.** 1996. Infection of human intestinal epithelial cells with invasive bacteria upregulates apical intercellular adhesion molecule-1 (ICAM-1) expression and neutrophil adhesion. J. Clin. Invest. **98:**572–583.
13. **Ilver, D., H. Källström, S. Normark, and A. B. Jonsson.** 1998. Transcellular passage of *Neisseria gonorrhoeae* involves pilus phase variation. Infect. Immun. **66:**469–473.
14. **Jarvis, G. A.** 1994. Analysis of C3 deposition and degradation on *Neisseria meningitidis* and *Neisseria gonorrhoeae*. Infect. Immun. **62:**1755–1760.
15. **Kashihara-Sawami, M., and D. A. Norris.** 1992. The state of differentiation of cultured human keratinocytes determines the level of intercellular adhesion molecule-1 (ICAM-1) expression induced by γ interferon. J. Investig. Dermatol. **98:**741–747.
16. **Kellogg, D. J., I. R. Cohen, L. C. Norins, A. L. Schroeter, and G. Reising.** 1968. *Neisseria gonorrhoeae*. II. Colonial variation and pathogenicity during 35 months in vitro. J. Bacteriol. **96:**596–605.
17. **Kilgore, K. S., J. P. Shen, B. F. Miller, P. A. Ward, and J. S. Warren.** 1995. Enhancement by the complement membrane attack complex of tumor necrosis factor-α-induced endothelial cell expression of E-selectin and ICAM-1. J. Immunol. **155:**1434–1441.
18. **King, G., J. F. James, and J. Swanson.** 1978. Studies on gonococcus infection. XI. Comparison of in vivo and vitro association of *Neisseria gonorrhoeae* with human neutrophils. J. Infect. Dis. **137:**38–43.
19. **Kupsch, E. M., B. Knepper, T. Kuroki, I. Heuer, and T. F. Meyer.** 1993. Variable opacity (Opa) outer membrane proteins account for the cell tropisms displayed by *Neisseria gonorrhoeae* for human leukocytes and epithelial cells. EMBO J. **12:**641–650.
20. **McGee, Z. A., C. M. Clemens, R. L. Jensen, J. J. Klein, L. R. Barley, and G. L. Gorby.** 1992. Local induction of tumor necrosis factor as a molecular mechanism of mucosal damage by gonococci. Microb. Pathog. **12:**333–341.
21. **McQuillen, D. P., D. B. Jani, and P. A. Rice.** 1991. Relationship of sialylation of lipooligosaccharide (LOS) of *N. gonorrhoeae* to cleavage of C3 and serum resistance, p. 353–358. *In* M. Achtman, P. Kohl, C. Marchal, G. Morelli, A. Seiler, and B. Thiesen (ed.), Neisseriae 1990. Walter de Gruyter, New York, N.Y.
22. **Merz, A. J., and M. So.** 1997. Attachment of piliated, Opa^- and Opc^- gonococci and meningococci to epithelial cells elicits cortical actin rearrangements and clustering of tyrosine-phosphorylated proteins. Infect. Immun. **65:**4341–4349.
23. **Naumann, M., S. Wessler, C. Bartsch, B. Wieland, and T. F. Meyer.** 1997. *Neisseria gonorrhoeae* epithelial cell interaction leads to the activation of the transcription factors nuclear factor κB and activator protein 1 and the induction of inflammatory cytokines. J. Exp. Med. **186:**247–258.
24. **Pettit, R. K., and R. C. Judd.** 1992. The interaction of naturally elaborated blebs from serum-susceptible and serum-resistant strains of *Neisseria gonorrhoeae* with normal human serum. Mol. Microbiol. **6:**729–734.
25. **Price, R. J., and B. Boettcher.** 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.
26. **Ramsey, K. H., H. Schneider, A. S. Cross, J. W. Boslego, D. L. Hoover, T. L. Staley, R. A. Kuschner, and C. D. Deal.** 1995. Inflammatory cytokines produced in response to experimental human gonorrhea. J. Infect. Dis. **172:** 186–191.
27. **Richardson, W. P., and J. C. Sadoff.** 1988. Induced engulfment of *Neisseria gonorrhoeae* by tissue culture cells. Infect. Immun. **56:**2512–2514.
28. **Rothlein, R., E. A. Mainolfi, M. Czajkowski, and S. D. Marlin.** 1991. A form of circulating ICAM-1 in human serum. J. Immunol. **147:**3788–3793.
29. **Rousset, M., G. Chevalier, J. P. Rousset, E. Dussaulx, and A. Zweibaum.** 1979. Presence and cell growth-related variations of glycogen in human colorectal adenocarcinoma cell lines in culture. Cancer Res. **39:**531–534.
30. **Rudel, T., I. Scheurerpflug, and T. F. Meyer.** 1995. Neisseria PilC protein identified as type-4 pilus tip-located adhesin. Nature **373:**357–359.
31. **Rudel, T., J. P. van Putten, C. P. Gibbs, R. Haas, and T. F. Meyer.** 1992. Interaction of two variable proteins PilE and PilC required for pilus-mediated adherence of *Neisseria gonorrhoeae* to human epithelial cells. Mol. Microbiol. **6:**3439–3450.
32. **Sellak, H., E. Franzini, J. Hakim, and C. Pasquier.** 1994. Reactive oxygen species rapidly increase endothelial ICAM-1 ability to bind neutrophils without detectable upregulation. Blood **83:**2669–2677.
33. **Shaw, J. H., and S. Falkow.** 1988. Model for invasion of human tissue culture cells by *Neisseria gonorrhoeae*. Infect. Immun. **56:**1625–1632.
34. **Staunton, D. E., S. D. Marlin, C. Stratowa, M. L. Dustin, and T. A. Springer.** 1988. Primary structure of ICAM-1 demonstrates interaction between members of the immunoglobulin and integrin supergene families. Cell **52:**925–933.
35. **Swanson, J., K. Robbins, O. Barrera, D. Corwin, J. Boslego, J. Ciak, M. Blake, and J. M. Koomey.** 1987. Gonococcal pilin variants in experimental gonorrhea. J. Exp. Med. **165:**1344–1357.
36. **Sykes, J. A., J. Whitescarver, P. Jernstrom, J. F. Nolan, and P. Byatt.** 1970. Some properties of a new epithelial cell line of human origin. J. Natl. Cancer Inst. **45:**107–122.
37. **Vanderpuye, O. A., C. A. Labarrere, and J. A. McIntyre.** 1992. The complement system in human reproduction. Am. J. Reprod. Immunol. **27:**145–155.
38. **van de Stolpe, A. and P. van der Saag.** 1996. Intercellular adhesion molecule-1. J. Mol. Med. **74:**13–33.
39. **Vaporciyan, A. A., M. S. Mulligan, J. S. Warren, P. A. Barton, M. Miyasaka, and P. A. Ward.** 1995. Up-regulation of lung vascular ICAM-1 in rats is complement dependent. J. Immunol. **155:**1442–1449.
40. **von Kleist, S., E. Chany, P. Burtin, M. King, and J. Fogh.** 1975. Immunohistology of the antigenic pattern of a continuous cell line from a human colon tumor. J. Natl. Cancer Inst. **55:**555–560.
41. **Wang, J., H. Schneider, and J. M. Griffiss.** 1996. *Neisseria gonorrhoeae* must express the paraglobosyl LOS in order to invade human genitourinary epithelial cells, p. 112–113. *In* W. D. Zollinger, C. E. Frasch, and C. D. Deal (ed.), Abstracts of the 10th International Pathogenic Neisseria Conference.
42. **Wong, H., W. D. Anderson, T. Cheng, and K. T. Riabowol.** 1994. Monitoring mRNA expression by polymerase chain reaction: the "primer-dropping" method. Anal. Biochem. **223:**251–258.

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