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Nuclear Factor-κB Directs Carcinoembryonic Antigen-related Cellular Adhesion Molecule 1 Receptor Expression in *Neisseria gonorrhoeae*-infected Epithelial Cells*

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The human-specific pathogen *Neisseria gonorrhoeae* expresses opacity-associated (Opa) protein adhesins that bind to various members of the carcinoembryonic antigen-related cellular adhesion molecule (CEACAM) family. In this study, we have analyzed the mechanism underlying *N. gonorrhoeae*-induced CEACAM up-regulation in epithelial cells. Epithelial cells represent the first barrier for the microbial pathogen. We therefore characterized CEACAM expression in primary human ovarian surface epithelial (HOSE) cells and found that CEACAM1–3 (L, S) and CEACAM1–4 (L, S) splice variants mediate an increased Opa_{52}-dependent gonoccocal binding to HOSE cells. Up-regulation of these CEACAM molecules in HOSE cells is a direct process that takes place within 2 h postinfection and depends on close contact between microbial pathogen and HOSE cells. *N. gonorrhoeae*-triggered CEACAM1 up-regulation involves activation of the transcription factor nuclear factor κB (NF-κB), which translocates as a p50/p65 heterodimer into the nucleus, and an NF-κB-specific inhibitory peptide inhibited CEACAM1-receptor up-regulation in *N. gonorrhoeae*-infected HOSE cells. Bacterial lipopolysaccharides did not induce NF-κB and CEACAM up-regulation, which corresponds to our findings that HOSE cells do not express toll-like receptor 4. The ability of *N. gonorrhoeae* to up-regulate its epithelial receptor CEACAM1 through NF-κB suggests an important mechanism allowing efficient bacterial colonization during the initial infection process.

During natural infections, pathogenic *Neisseria* species primarily colonize epithelial cells of the human nasopharynx or urogenital tract. Among the virulence factors involved in mucosal colonization, the colony opacity-associated (Opa)[1] proteins are believed to play an important role as bacterial adhesins and invasins. The Opa proteins are a family of functionally and antigenically diverse outer membrane proteins. The gonococcal chromosome contains up to 11 unlinked alleles encoding distinct Opa variants (1). Each variant is independently regulated by phase variation, resulting in a heterogeneous population of bacteria expressing no, one, or several Opa variants (2). Bacteria recovered from natural infections and following inoculation of human volunteers with Opa^- bacteria are mostly Opa^+, suggesting an important role for the Opa adhesins during infection (3, 4). Using recombinant *Escherichia coli* and *Neisseria gonorrhoeae* strains, we have previously characterized the receptor specificities of all Opa variants of the MS11 strain of *N. gonorrhoeae* (1, 5). These and other studies have revealed that a minority of Opa proteins can target *Neisseria* species to heparan sulfate proteoglycan receptors (6, 7) and, via binding to vitronectin and fibronectin, to cell surface integrins (8, 9). Most Opa variants characterized to date interact with the family of human carcinoembryonic antigen-related cellular adhesion molecules (5, 10–15). Whereas some Opa proteins may interact with both heparan sulfate proteoglycan and CEACAM receptors (12, 16), each variant appears to mediate host cell invasion only via either one or the other receptor class. These different binding specificities may have important implications for the pathogenic process of *Neisseria*, since the distribution pattern of each CEACAM receptor should influence the cellular tropism of neisserial strains expressing different Opa variants *in vivo*. In addition, very different cellular processes have been linked to individual CEA family members (17–21), suggesting that the cellular response to neisserial binding depends upon the specific combination of CEACAM receptors engaged on certain cell types.

CEACAM1 (BGP, CD66a), CEACAM3 (CGM1, CD66d), CEA (CD66e), and CEACAM6 (NCA, CD66c) (see Ref. 22 for changes in nomenclature) serve as receptors for the pathogenic *Neisseria* species (5, 10, 12, 13). All of these receptors can mediate gonococcal invasion when recombinantly expressed in a CEACAM-negative epithelial cell line (5, 12, 13) The closely related molecules CEACAM4, CEACAM7, and CEACAM8 are not recognized by any Opa variants tested to date (23). Each CEACAM receptor consists of an immunoglobulin variable-like domain followed by a variable number of IgC2 constant-like domains (17). CEA, CEACAM6, CEACAM7, and CEACAM8 are glycosylphosphatidylinositol-linked to the cell surface, whereas CEACAM1 and CEACAM3 are inserted into the cellular membrane via a carboxyl-terminal transmembrane and cytoplasmic domain (24–27). Despite the fact that each receptor is highly glycosylated, binding is a protein-protein interaction with Opa proteins recognizing CEACAM residues exposed on the GFCC′ face of the amino-terminal domain (28). Several

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[1] The abbreviations used are: Opa, opacity-associated; CEACAM, carcinoembryonic antigen-related cellular adhesion molecule; PMN, polymorphonuclear neutrophil; HUVEC, human umbilical vein endothelial cell; LPS, lipopolysaccharide; TLR, toll-like receptor; NF-κB, nuclear factor κB; HOSE, human ovarian surface epithelial; TNF, tumor necrosis factor; FACS, fluorescence-activated cell sorting; RT, reverse transcription; ELISA, enzyme-linked immunosorbent assay; mAb, monoclonal antibody; asialo GM1, gangliotetraosylceramide (Galβ1,3GalNAcβ1,4Galβ1,4Glcβ1,1Cer).

different splice variants of CEACAM1 and CEACAM3 exist. All contain the N-terminal domain but differ in the number of extracellular IgC-like domains and the length of their cytoplasmic domains (29, 30).

CEACAM receptors mediate intercellular adhesion via both homotypic (CEACAM1, CEA, and CEACAM6) and/or heterotypic (CEA-CEACAM6 and CEACAM6-CEACAM8) interactions (31, 32). It has been demonstrated that the N domain is directly involved in the cell adhesion phenomena. CEACAM1 and CEACAM6 are also involved in the adherence of activated neutrophils to cytokine-activated endothelial cells, both directly through their ability to present the sialylated Lewis^x antigen to E-selectin and indirectly by the CEACAM6-stimulated activation of CD18 integrins (18). The role of CEACAM receptors is not, however, restricted to simple anchorage to adjacent cells, since various receptors can influence cell cycle control and cellular differentiation. For example, CEACAM1 expression inhibits the proliferation of mouse colonic carcinoma cells both *in vitro* and *in vivo*. This effect was abrogated by deleting the receptor's cytoplasmic domain, suggesting an important role for CEACAM1-mediated signaling in this event (33, 34). Such a growth-inhibitory effect is consistent with clinical observations that CEACAM1 expression is down-regulated in various colonic carcinomas (35, 36). Altogether, these features imply an important role for members of the CEACAM receptor family as sensory and regulatory molecules in cell-cell adhesion events (37).

CEACAM1, CEACAM3, and CEACAM6 are expressed by human polymorphonuclear neutrophils (PMNs) and can mediate gonococcal binding and opsonin-independent phagocytosis by these phagocytes (10, 11, 14, 38). This interaction appears to play a central role in the pathogenic process, since a urethral exudate consisting primarily of PMNs associated with both intracellular and extracellularly attached gonococci is the hallmark of gonorrhea. CEACAM receptors expressed by other cells also appear to play an important role during other stages of neisserial infection. Polarized T84 epithelial cells express CEACAM1, CEA, and CEACAM6 on their apical surface, and Opa binding to these receptors mediates bacterial uptake, cellular transcytosis, and release at the basolateral surface (39). This is consistent with previous findings that *N. gonorrhoeae* and *N. meningitidis* appear in the subepithelial layers following the *in vitro* infection of organ cultures (40).

We could previously show that *N. gonorrhoeae* induces directly CEACAM1 receptor expression in human umbilical vein endothelial cells (HUVECs) (41). In the endothelial cell model, expression of CEACAM1 was induced by LPS via a toll-like receptor-4 (TLR-4)-dependent activation of nuclear factor κB (NF-κB). This increased expression of CEACAM1 correlates with an increased adherence and invasion of different Opa-expressing bacteria into these cells *in vitro* (5, 15, 41). We were therefore interested in determining whether gonococci were also capable of inducing their cellular receptor in epithelial cells that form the first mechanical barrier and primary site of infection. In the present study, we demonstrate that *N. gonorrhoeae* infection stimulates directly CEACAM1 receptor expression in human ovarian surface epithelial (HOSE) cells. We are able to show that up-regulation of CEACAM1 expression in HOSE cells is not due to an autocrine loop via TNFα. As in HUVECs, CEACAM1 expression is regulated through activation of NF-κB. However, in contrast to HUVECs, up-regulation of CEACAM1 in HOSE cells is an LPS/TLR-4-independent event that critically depends on a direct interaction of gonococci with HOSE cells. The newly expressed CEACAM1 receptor allows gonococci to establish a tight, Opa-dependent anchorage to the epithelia and may lead to bacterial uptake into the target cells.

MATERIALS AND METHODS

Cell Lines—Primary HOSE cells that had been isolated and immortalized as previously described (42) were grown in M199 and MCDB-104 medium (1:1) (Invitrogen) with 10% heat-inactivated fetal calf serum in a humidified atmosphere at 37 °C with 5% CO_2. Epithelial cells were grown to form a confluent monolayer and then seeded to new flasks or into wells containing glass coverslips to obtain a confluence of about 60%. HUVECs were obtained from human umbilical vein by chymotrypsin digestion as described previously (43), cultured in low serum endothelial growth medium (PromoCell, Heidelberg, Germany), and used between passages 4 and 5. Human PMNs were isolated from venous blood of healthy donors as described previously (44). The construction of stably transfected HeLa cell lines expressing CEA and CEACAM1 was described previously (45).

Bacterial Strains—The strains of *N. gonorrhoeae* MS11 expressing defined recombinant Opa variants were described previously (1). Strains N309 and N313 invariantly synthesize the CEACAM-binding Opa proteins Opa_{52} and Opa_{57}, respectively. Strain N303 expresses the heparan sulfate-binding Opa_{50}. In these strains, the cloned *opa* genes were expressed in the genetic background of the MS11 derivative N279, which lacks pili and carries a deletion in the epithelial cell invasion-associated *opaC30* locus. N280 is a piliated variant of N279 (Opa^-, P^+) (1). Daily subculture of all strains was carried out using a binocular microscope to select for desired Opa phenotypes, and Opa protein expression was verified by SDS-PAGE and immunoblot using the monoclonal antibody 4B12C11 (46) for the detection of Opa proteins.

Bacterial Infection Assays and Stimulation of HOSE Cells—For infection experiments, HOSE cells were seeded into 75-cm^2 flasks to obtain cultures at about 70% confluence at the time of infection. One night before infection, the medium was changed to M199 medium (Invitrogen) supplemented with 2% heat-inactivated fetal calf serum. Gonococci were harvested from fresh overnight cultures into M199 medium containing 2% heat-inactivated fetal calf serum to obtain a culture density of 10^8 colony-forming units/ml and then used to infect the cells at a multiplicity of infection of 30 bacteria/cell for the indicated periods. For immunofluorescence analysis, HOSE cells were infected as outlined above, except that cells were initially seeded onto 12-mm glass coverslips, and the samples were fixed after the final washing step postinfection by incubating in 3.7% paraformaldehyde in 200 mM HEPES buffer (pH 7.4) for 30 min at room temperature. To determine levels of gonococcal adherence and invasion, the gonococci were stained for immunofluorescence and then analyzed by confocal laser-scanning microscopy as described previously (15, 39). The visualization of bacterial interactions with the CEACAM1 receptor was also analyzed by confocal laser microscopy using the anti-CEACAM mouse monoclonal antibody D14HD11. Fluorescein isothiocyanate-conjugated goat anti-mouse and goat anti-rabbit antibodies (Dianova) were used as secondary antibodies. Tetramethylrhodamine isothiocyanate-phalloidin was used to visualize cellular actin. To quantitate total cell-associated bacteria in plating assays, cells were infected in 24-well plates. After the infection periods indicated, monolayers were washed three times with 1 ml of medium and lysed with 1% saponin in M199 for 10 min. Gonococci were suspended by vigorous pipetting, and colony-forming units in the lysates were determined by plating of serial dilutions. A polyclonal rabbit antiserum raised against human carcinoembryonic antigen (A0115) and control rabbit immunoglobulins (X0936) were purchased from DAKO (Glostrup, Denmark), and immunoglobulins were purified over a protein G column. To block CEACAM receptors, cells were incubated with purified specific or control immunoglobulins at 20 μg/ml for 30 min at 37 °C before infection. Where indicated, cells were stimulated with TNFα (BD PharMingen, San Diego) or with LPS prepared from *E. coli* serotype O111:B4 by phenol extraction (Sigma). Suspensions of LPS were prepared by sonication in endotoxin-free water (Invitrogen) to disperse any aggregates formed and were then diluted to the indicated final concentration in supplemented medium.

FACS Analysis—HOSE cells were analyzed using a FACS-Calibur (Becton Dickinson, Heidelberg, Germany) and the Cellquest software (Becton Dickinson). The CEACAM-specific, mouse-derived monoclonal antibodies (mAbs) 4/3/17 (anti-CEACAM1/CEA), 9A6 (anti-CEACAM6), and COL1 (anti-CEA/CEACAM3) were used for primary labeling and were described previously (47–49). The background fluorescence was determined using isotype-matched mouse IgG as a negative control.

Immunoblotting—CEACAM1 protein expression in response to exposure to bacterial strains, TNFα, or other stimuli was determined by immunoblot analysis of total cellular protein essentially as described before (15). Protein concentration in each sample was determined by colorimetric Bradford protein assay (Bio-Rad), and equal amounts of

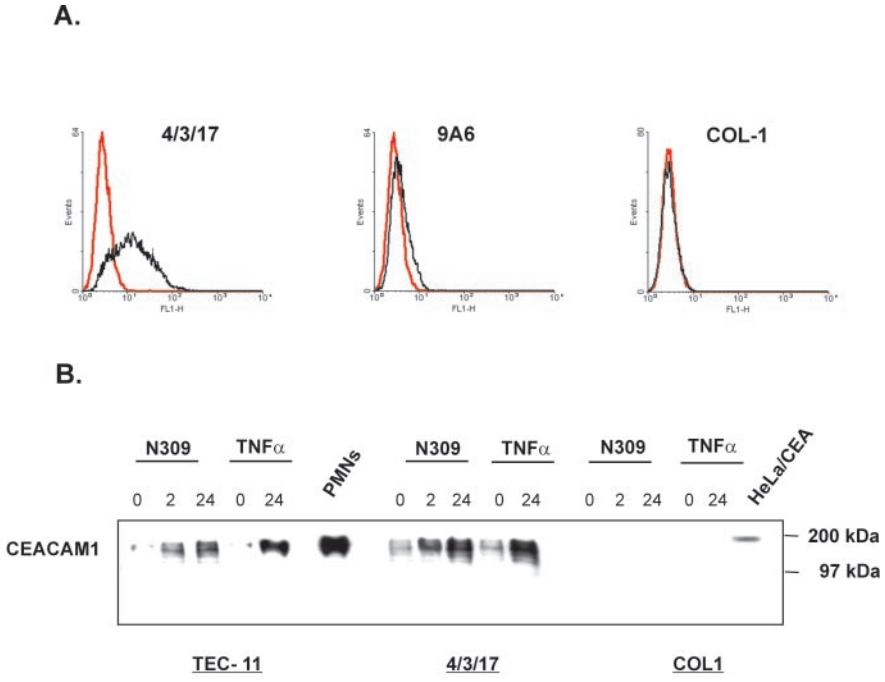

FIG. 1. **Characterization of the CEACAM expression pattern on ovarian surface epithelial cells (HOSE).** *A*, FACS analysis of CEACAM expression pattern. Staining with the cross-specific mAb 4/3/17 (CEACAM1/CEA) shows expression of CEACAM1 (*left panel*), since staining with another cross-specific mAb, COL-1 (CEA/CEACAM3), was negative (*right panel*). Little amounts of CEACAM6 were detected using the specific mAb 9A6. Dead cells were excluded from the histograms by staining with propidium iodide. *Red lines*, isotype control; *black lines*: anti-CEACAM antibodies. *B*, characterization of the CEACAM expression pattern using Western blot analysis. HOSE cells were either infected with N309 (Opa_{52}) or stimulated with 10 ng/ml TNFα. At the indicated periods of time, the cell lysates were harvested and analyzed by Western blot analysis using the CEACAM1 receptor-specific monoclonal antibody TEC-11 or the cross-specific monoclonal mAbs 4/3/17 and COL-1. PMNs and HeLa cells stably transfected with CEA were used as controls.

protein were separated by SDS-PAGE and blotted onto Immobilon P transfer membranes (Millipore Corp.). Western blot analysis was performed using the CEACAM1-specific monoclonal antibody TEC-11 (50); the CEACAM1, CEACAM3, CEA, and CEACAM6 cross-specific monoclonal antibody D14HD11; the CEACAM6-specific antibody 9A6 (Immunotech, Marseille, France); the CEACAM1 and CEA cross-specific antibody 4/3/17; and the CEA and CEACAM3 cross-specific antibody COL1. Bound antibodies were detected using a peroxidase-conjugated goat anti-mouse secondary antibody and the ECL chemiluminescence detection system (Amersham Pharmacia Biotech). To test for I$\kappa\beta\alpha$ degradation, cytosolic fractions obtained from HOSE cells exposed to various stimuli were analyzed by immunoblot analysis using an I$\kappa\beta\alpha$−specific polyclonal antibody that does not cross-react with other I$\kappa\beta$ family members (C-21; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Coomassie Blue-stained gels were generally used as a control to assure that equal protein amounts were applied (data not shown).

ELISA—Cytokines were assayed in the supernatants of *Neisseria*-infected HOSE cells at different time points. TNFα ELISAs were performed as described in the manufacturer's instructions (ELISA BIOSOURCE Europe S.A.).

Inhibitor Experiments—NF-κB SN50 (BIOMOL Research Laboratories, Inc.) is a cell-permeable peptide that inhibits the translocation of active NF-κB complex into the nucleus. To confirm the role of NF-κB in CEACAM expression, cells were pretreated with 50 μg/ml of this peptide for 30 min at 37 °C before TNFα or the bacteria was added.

Reverse Transcription (RT)-PCR Analysis—Total RNA was isolated from HOSE cells that had been treated with various stimuli, as indicated, using either the SV Total RNA Isolation System (Promega) or the Qiagen RNeasy Kit, as outlined by the manufacturers, and then treated further with RNase-free DNase I. Equal amounts of RNA were reverse transcribed into single-stranded cDNA using Superscript IIRT (Invitrogen) and oligo(dT) primers. As a control for chromosomal DNA contamination, RNA was used directly for PCR amplification. Subsequent amplification of CEACAM1 was carried out using CEACAM1-specific primers for 30 cycles at 56 °C annealing temperature. The differential amplification of CEACAM1 splice variants was performed using *Taq* polymerase (Invitrogen) for 30 cycles with an annealing temperature of 56 °C. The primers used were 5′ (ACAGTCAAGACGATCATAGT) and 3′ (ATCTTGTTAGGTGGGTCATT), resulting in amplified fragments in the DNA sequence as previously described (51). To detect Toll-like receptor expression, PCR amplification of the cDNA template was performed using *Taq* polymerase for 28 cycles at 95 °C for 40 s, 54 °C for 40 s, and 72 °C for 1 min. PCR primers used for TLR-2 were GCCAAAGTCTCTTGATTGATTCC and TTGAAGTTCTCCAGCTCCTG, and those used for TLR-4 were TGGATACGTTTCCTTATAAG and GAAATGGAGGCACCCCTTC (52). Primers specific for the constitutively expressed housekeeping gene β-actin were also included within the reaction mixture to provide an internal control that allowed samples to be equally loaded. In each case, PCR products were visualized by ethidium bromide staining after agarose gel electrophoresis.

Electrophoretic Mobility Shift Assay—After the indicated periods of infection, cytoplasmic and nuclear extracts were prepared using the nonionic detergent method described previously (53). Gel retardation assays for the detection of the active NF-κB complex were performed with an Igκ oligonucleotide that had been labeled using the large fragment DNA polymerase (Klenow) in the presence of [α-^{32}P]deoxy-ATP. The DNA-binding reactions were performed in 20 μl of binding buffer for 20 min at 30 °C. Competition experiments and supershift assays were performed with antibodies as previously described (53). The reaction products were analyzed by electrophoresis in a 5% polyacrylamide gel using 12.5 mM Tris, 12.5 mM boric acid, and 0.25 mM EDTA, pH 8.3. Gels were then dried and exposed to Amersham TM films (Amersham Biosciences, Inc.) at −70 °C using an intensifying screen.

RESULTS

Characterization of the CEACAM Expression Pattern on HOSE Cells—In search of a suitable human cell line to study CEACAM1 regulation, we first examined CEACAM receptor expression by various carcinoma cell lines commonly used to study *Neisseria* host cell interactions. A HeLa cervix carcinoma cell line and a HEC-1-B endometrial carcinoma line were found to be negative for CEACAM expression and unresponsive to TNFα, whereas ME-180 cervix carcinoma cells produced high levels of CEA and CEACAM6 but no CEACAM1. These findings were consistent with the frequently observed dysregulation of CEACAM expression in carcinoma cell lines and further suggested that primary epithelial cells would be a more suitable model to study transcriptional regulation of CEACAM1 expression. We therefore characterized CEACAM expression in HOSE cells that were derived from primary human ovarian epithelium and immortalized by a retroviral vector expressing human papilloma viral oncogenes E6 and E7 (42).

The CEACAM expression pattern of HOSE cells was analyzed by FACS analysis (Fig. 1*A*) using the monoclonal antibodies 4/3/17 (anti-CEACAM1/CEA), 9A6 (anti-CEACAM6), and COL-1 (anti-CEACAM3/CEA). COL-1 did not label HOSE cells, showing that they expressed neither CEACAM3 nor CEA. The clear peak detected by the cross-specific mAb 4/3/17 therefore represents CEACAM1. The mAb 9A6 additionally detected very low level expression of CEACAM6. These results were

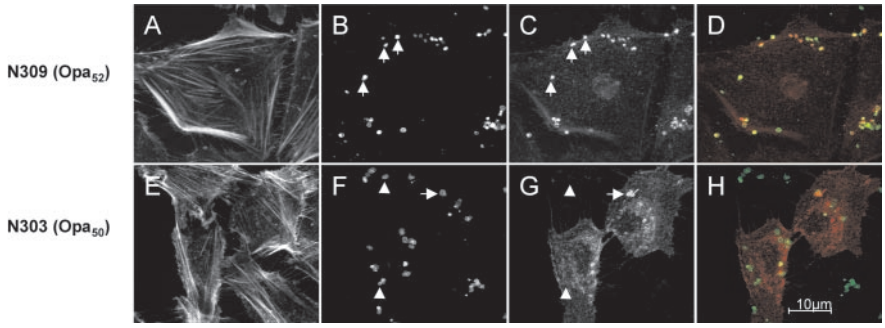

FIG. 2. **Co-localization of CEACAM antigens and Opa-expressing *N. gonorrhoea* on the surface of HOSE cells.** HOSE cells were seeded on glass coverslips and infected with *N. gonorrhoeae* N309 expressing Opa_{52} (*A–D*) or N303 expressing Opa_{50} (*E–H*). After 1 h, infected cells were washed, fixed, and stained using phalloidin to visualize cellular actin (*A* and *E*), rabbit antiserum against gonococci (*B* and *F*), and a monoclonal antibody against CEACAM1 (*C* and *G*). *D* and *H* show pseudocolored overlays of fluorescence signals obtained from CEACAM1 (*red*) and anti-gonococcal antiserum (*green*). Adherence by the CEACAM1-binding strain N309 resulted in marked receptor recruitment by all adhering bacteria (*arrows* in *B* and *C*). Strain N303 adhered irrespective of CEACAM1 expression and did not recruit the CEACAM1 receptor (*arrowheads* in *F* and *G*), which remained evenly distributed over the cell surface. The *arrows* in *F* and *G* point to an exceptional bacterium that did recruit CEACAM1, probably due to the phase-variable expression of a CEACAM-binding Opa in addition to Opa_{50}. Images are projections of four or five confocal sections taken every 0.5 μm to visualize total cellular receptor and all adhering bacteria.

confirmed by Western blot analysis (Fig. 1*B*). Cellular lysates were prepared at different time points after infection with N309 (Opa_{52}) or stimulation with TNFα. PMNs, expressing CEACAM1, CEACAM3, and CEACAM6, and HeLa cells stably transfected with CEA were used as controls. Expression of CEACAM1 was detected using the mAb TEC-11, which specifically recognizes the A2 domain that is found exclusively in some splice variants of CEACAM1. This result was verified by using the cross-reactive N domain mAb 4/3/17, which recognizes both CEACAM1 and CEA. Western blot analysis confirmed that CEACAM1 is expressed on HOSE cells. Furthermore, the CEACAM1 receptor expression was found to be up-regulated during neisserial infection and stimulation with TNFα. The significant up-regulation of CEACAM1 receptor expression starts already 2 h after infection (Fig. 1*B*).

Interaction of Opa Variants with HOSE Cells—To further characterize HOSE cells as a model cell line for Opa-mediated gonococcal infection, cells were infected with isogenic strains expressing defined and functionally distinct Opa variants. Infected cells were fixed after 30 min, followed by immunocytochemical staining and confocal laser-scanning microscopy. As illustrated in Fig. 2, HOSE cells infected with N309 (Opa_{52}) and N303 (Opa_{50}) showed an equally strong adherence, whereas the association of N302 (Opa^-) gonococci was only very weak (data not shown). Only Opa_{52}-expressing gonococci recruited CEACAM1, resulting in a strong co-localization of the bacteria with CEACAM1 molecules that is typical of CEACAM-mediated adherence. In contrast, *N. gonorrhoeae* expressing the heparan sulfate-binding Opa_{50} variant (5–7) adhered to HOSE cells independently of their level of CEACAM1 expression and generally failed to recruit the receptor. Individual Opa_{50}-expressing gonococci co-localizing with CEACAM1 were occasionally observed. However, CEACAM receptor recruitment by these bacteria may have occurred as a result of phase variation, leading to the expression of a CEACAM-binding Opa variant from a chromosomal locus in addition to Opa_{50}.

To determine whether the induced CEACAM1 expression resulted in an increased bacterial binding, HOSE cells were infected with various gonococcal strains, and total adhering and intracellular bacteria per cell were quantified by confocal laser-scanning microscopy. We found that extended infection resulted in steadily increasing levels of Opa-mediated bacterial binding to otherwise unstimulated HOSE cells, and this correlated with an increased level of bacterial invasion (Fig. 3*A*). Interestingly, despite strong gonococcal adherence to HOSE cells, invasion was much lower when compared with endothelial cells as previously described (41). Opa-negative gonococci also bound to HOSE cells but generally in much lower numbers, and invasion was minimal (Fig. 3*B*).

In a plating assay, the adherence of two isogenic gonococcal strains expressing different CEACAM-binding Opa variants, Opa_{52} and Opa_{57} (5), was specifically inhibited by rabbit immunoglobulins raised against human carcinoembryonic antigen but not by control immunoglobulins (Fig. 3*C*). In marked contrast, neither adherence via the heparan sulfate-binding Opa_{50} adhesin of strain N303 (5) nor attachment of an Opa-negative strain expressing type IV pili (N280) was sensitive to inhibition. These data confirm the critical role of CEACAM1 as a receptor for Opa_{52} and Opa_{57} (N313)-expressing gonococci in the HOSE cell model while highlighting the additional existence of different cellular receptors for other gonococcal adhesins (*i.e.* Opa_{50} and type IV pili) that are known not to interact with CEACAM1 (5).

Next we sought to determine whether increased adhesion of Opa_{52}-expressing bacteria to the HOSE cells observed during time course experiments (Fig. 3*A*) reflected increasing CEACAM1 expression levels or whether it was primarily due to proliferation of cell-associated bacteria. To assess the effect of receptor expression independently, HOSE cells were pretreated with or without TNFα, infected with N309 (Opa_{52}), and analyzed by plating of cell-associated bacteria (Fig. 4*A*). TNFα-induced up-regulation of CEACAM1 resulted in significantly stronger bacterial adherence at all time points tested. This effect was most readily observed early, within 30 min of infection, when TNFα pretreatment increased the number of adhering bacteria by more than 10-fold. Opa-negative bacteria still failed to adhere to HOSE cells after TNFα treatment, whereas attachment of strain N309 remained fully sensitive to specific blockade by anti-CEA immunoglobulins (Fig. 4*B*). Taken together, these data demonstrate that also after TNFα treatment of HOSE cells, adhesion of strain N309 was strictly dependent on the Opa-CEACAM interaction.

We have shown previously that gonococcal infection can induce TNFα production by epithelial cells (53). Therefore, an autocrine loop involving *de novo* TNFα expression that leads to the subsequent induction of CEACAM1 expression could presumably explain the increased gonococcal binding seen in Figs. 3*A* and 4. To assess secretion of TNFα in response to gonococcal infection of HOSE cells, TNFα in culture supernatants was assayed in an ELISA at different time points after infection. The secretion of TNFα was very low, and also 24 h after infection it was almost undetectable (data not shown).

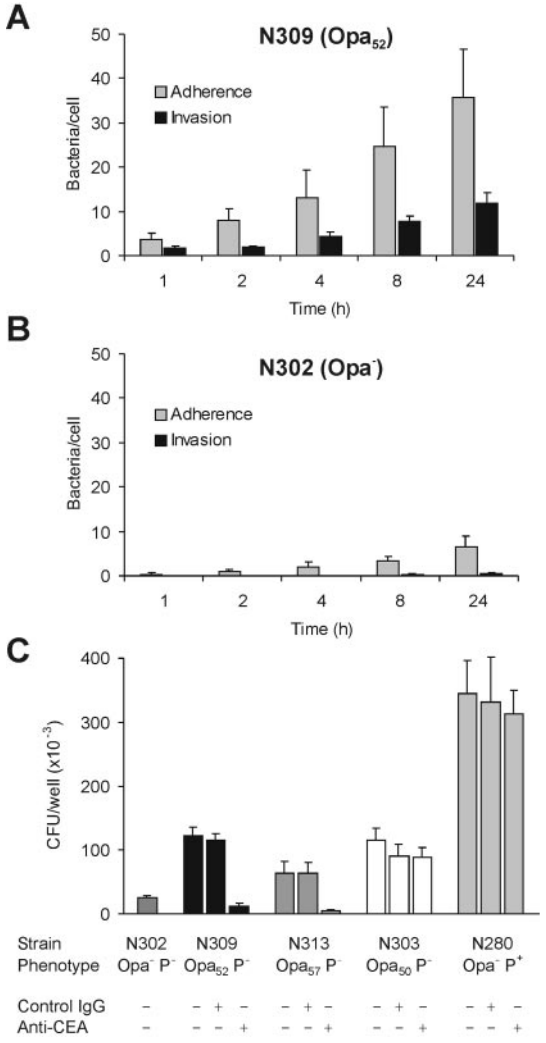

FIG. 3. **Gonococcal adherence to HOSE cells via Opa_{52} increases with time and depends on the Opa-CEACAM interaction.** HOSE cells seeded on glass coverslips were infected with N309 (Opa_{52}) (*A*) or with N302 (Opa^-) (*B*) for 1–24 h. At the indicated time points, cells were fixed and then labeled for immunofluorescence analysis by confocal laser-scanning microscopy. Total associated and intracellular bacteria per cell were counted. *Gray bars* show adherence to HOSE cells; *black bars* show intracellular bacteria associated with HOSE cells. Assays were performed in triplicate on at least three separate occasions, and data illustrate the mean ± S.D. of one representative experiment. *C*, plating assay to determine the role of CEACAM1 in adherence of gonococcal strains expressing functionally diverse adhesins. HOSE cells were pretreated with 20 μg/ml anti-CEA rabbit immunoglobulins or nonspecific control immunoglobulins and infected with a nonadhering, Opa-negative strain (N302), with strains expressing CEACAM-binding Opa variants (N309 and N313), with a strain expressing the heparan sulfate-binding Opa_{50} (N303) or with an Opa-negative strain expressing type IV pili. After 4 h, infected cultures were washed, and cell-associated bacteria were quantitated by dilution plating after disruption of eukaryotic cell membranes with 1% saponin in PBS. Data shown are representative of two independently performed experiments.

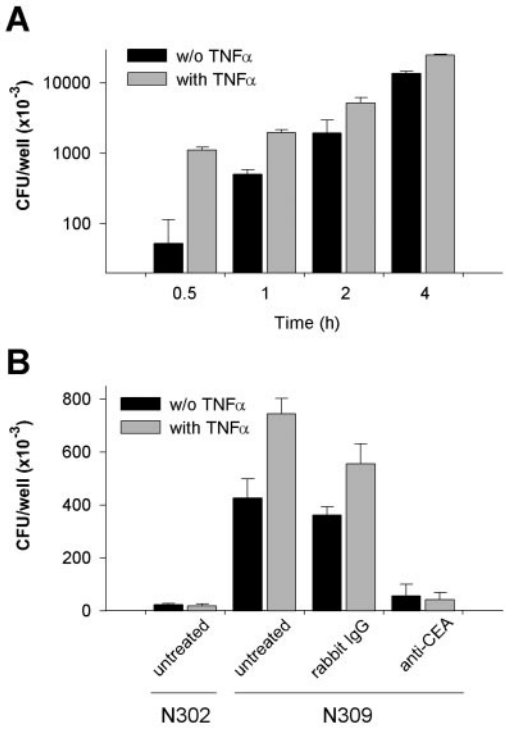

FIG. 4. **Increased neisserial adherence to HOSE cells following up-regulation of CEACAM1.** *A*, HOSE cells seeded in 24-well plates were prestimulated with 10 ng/ml TNFα for 12 h (*gray bars*) or left untreated (*black bars*). HOSE cells were then infected with Opa_{52}-expressing *N. gonorrhoeae* (N309) for 30 min to 4 h, and cell-associated bacteria were determined by dilution plating. Assays were performed in triplicate on at least three separate occasions, and data displayed illustrate the mean ± S.D. of one representative experiment. *B*, to determine whether increased adherence following stimulation by TNFα was dependent on the Opa-CEACAM interaction, HOSE cells were stimulated or not as above and infected for 1 h in the presence or absence of 20 μg/ml specific anti-CEA rabbit immunoglobulins or control immunoglobulins. Data shown are from one of two experiments with similar results.

Neisseria-induced CEACAM1 Expression Is Dependent upon Bacteria-Epithelial Cell Contact—To determine whether the increased CEACAM1 expression in HOSE cells depends on intimate contact between bacteria and cells, we compared receptor expression in the presence of adherent and nonadherent gonococci. HOSE cells were either left untreated, infected with isogenic neisserial strains expressing different Opa variants, or stimulated with TNFα as a positive control. Expression of the CEACAM1 protein was found to be induced rapidly during neisserial infection (Fig. 5*A*). Interestingly, efficient up-regulation of CEACAM1 expression required bacterial adherence, since induction by the Opa-negative and nonadherent strain N302 was much lower and occurred much later as compared with strongly adherent strains. However, the molecular mechanism of adherence was not important, since strains expressing either the CEACAM1-binding Opa_{52} or the heparan sulfate-binding Opa_{50} both induced CEACAM1. The rapid induction of CEACAM1 expression following infection was also confirmed by semiquantitative RT-PCR to detect CEACAM1-encoding transcript level (Fig. 5*B*). This shows that the CEACAM1 receptor up-regulation in HOSE cells requires bacterial adherence.

We generally observed increased levels of three defined protein bands by immunoblot analysis using the CEACAM receptor-specific monoclonal antibody D14HD11 (Fig. 5*A*). This expression pattern probably results from a combination of the variable glycosylation of CEACAM1 and/or the expression of multiple splice variants (54).

Expression of CEACAM1 Splice Variants by Human Epithelial Cells—Thirteen different CEACAM1 splice variants are known to exist. To analyze which splice variant(s) are induced in HOSE cells, we performed RT-PCR experiments with RNA from unstimulated and gonococcal-infected HOSE cells. As a positive control, endothelial cells (HUVECs) infected with N309 (Opa_{52}) were used. The primer pair used amplifies the mRNA fragment that spans from the middle of the Ig constant domain-like B1 region to the carboxyl-terminal end of the cytoplasmic domain. Using these primers, it is possible to dis-

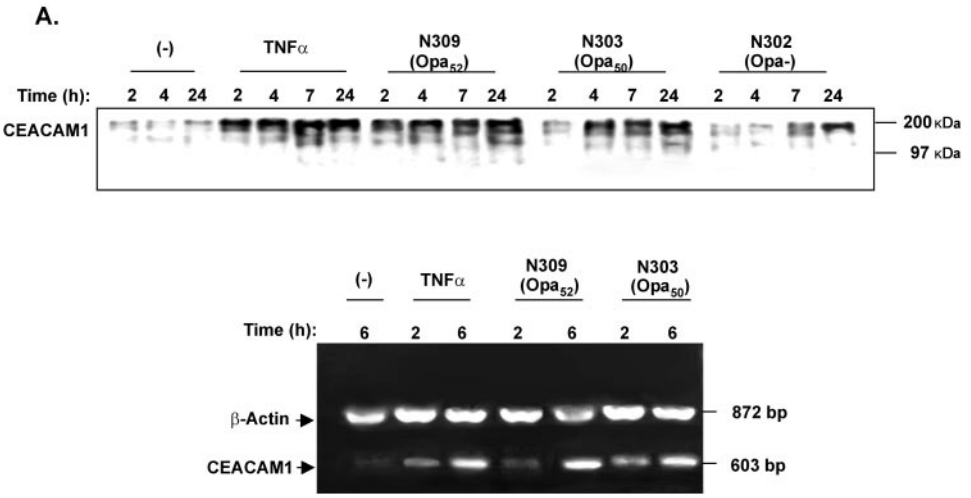

FIG. 5. **Effect of *N. gonorrhoeae* infection on CEACAM expression in HOSE cells.** *A*, HOSE cells were either left untreated, infected with different gonococcal strains, or stimulated with 10 ng/ml TNFα. Total protein was isolated at the time points indicated and separated by SDS-PAGE, and immunoblots were probed with the CEACAM receptor-specific monoclonal antibody D14HD11. *B*, *CEACAM1* transcript expression by HOSE. Total RNA was isolated after the indicated time intervals following the addition of TNFα or gonococcal infection and then reverse transcribed into single-stranded cDNA. Amplification of DNA was carried out by PCR using a *CEACAM1*-specific primer pair. In each case, transcript encoding β-actin was co-amplified as an internal control to assure that equal amounts of samples were applied.

criminate between known splice variants according to the size of the RT-PCR products (48). Epithelial cells were found to express four of the 13 known splice variants, as shown in Fig. 6. Two large RT-PCR products corresponded well with the 531- and 477-bp products expected from splice variants containing a complete set of four extracellular immunoglobulin-like domains and either a long (CEACAM1–4L; BGPa) or a short (CEACAM1–4S; BGPc) cytoplasmic domain, respectively. A pair of smaller RT-PCR products was consistent with extracellular A2 domain, again containing either a long cytoplasmic domain (CEACAM1–3L; BGPb) or not (CEACAM1–3S; BGPd). Both long splice variants (CEACAM1–4L and -3L) were expressed in significant amounts, whereas the expression levels of both short splice variants appeared to be much weaker (Fig. 6). Each long splice variant contains both the amino-terminal domain, which is bound by Opa proteins, and the long cytoplasmic domain, which contains the immunoreceptor tyrosine-based inhibitory motif-like sequences (22). The same pattern of splice variants was observed when the HOSE cells were infected with *N. gonorrhoeae* N303 (Opa_{50}) or N309 (Opa_{52}). The same expression pattern was also observed in HUVECs. In our previous experiments, we showed only two of the 13 splice variants, CEACAM1–4L and CEACAM1–3L (41), which could be explained by the fact that the cells were isolated from different donors, and the alternative splicing process might differ from donor to donor. To verify that the additional splice variants are not due to PCR artifacts, a stably transfected HeLa cell line expressing only CEACAM1–4L (BGPa) was analyzed (Fig. 6). In contrast to HOSE cells, the stably transfected HeLa cells express only one PCR product corresponding to the size of 531 bp (CEACAM1–4L).

NF-κB Directs CEACAM1 Expression in N. gonorrhoeae-infected Epithelial Cells—To address whether CEACAM1 expression depends on NF-κB activation, as has been reported previously (41), we infected subconfluent monolayers of HOSE cells with *N. gonorrhoeae* expressing either the heparan sulfate proteoglycan-specific Opa_{50}, the CEACAM-specific Opa_{52}, or no Opa protein, or cells were treated with TNFα. At different time points after challenge, the cells were harvested, and the nuclear fraction was prepared. The nuclear protein extracts were then analyzed for the levels of DNA binding activity in an

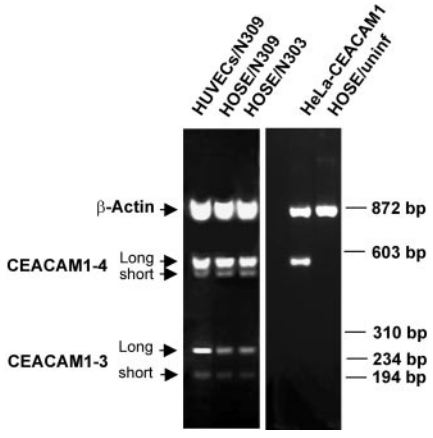

FIG. 6. **Expression of the CEACAM1 splice variants by HOSE cells.** HOSE cells were either left untreated or infected with gonococcal strains N309 (Opa_{52}) or N303 (Opa_{50}). HUVECs infected with N309 were used as a control. HeLa cells stably transfected with a cDNA for CEACAM1–4L should not contain splice variants and were used as an additional control (*right panel*). Total RNA was isolated after 2 h and reverse transcribed into cDNA. The expression of CEACAM1 splice variants was assessed by semiquantitative PCR amplification from the resulting template. The co-amplification of β-actin transcript was used as an internal control to confirm that equal amounts of cDNA were applied. These data are representative for at least three independent experiments.

electrophoretic mobility shift assay with a radioactively labeled oligonucleotide corresponding to the DNA-binding site of NF-κB (Fig. 7*A*). Protein binding of the oligonucleotide was observed within 90 min postinfection by N303 (Opa_{50}) and the N309 (Opa_{52}) strain, whereas NF-κB activation in response to the Opa-negative strain was very weak. TNFα treatment of the HOSE cells resulted in rapid translocation of NF-κB into the nucleus, with strong binding being observed within 10 min postinfection. Using the unlabeled oligonucleotide with the consensus sequence in a competition experiment, we confirmed the specificity of the binding activity (Fig. 7*A*). The molecular nature of the activated transcription factor complex was characterized using NF-κB-specific antibodies in a supershift assay (Fig. 7*A*). Nuclear extracts were preincubated with either anti-p50, anti-p65, anti-c-Rel, or preimmune serum before the ad-

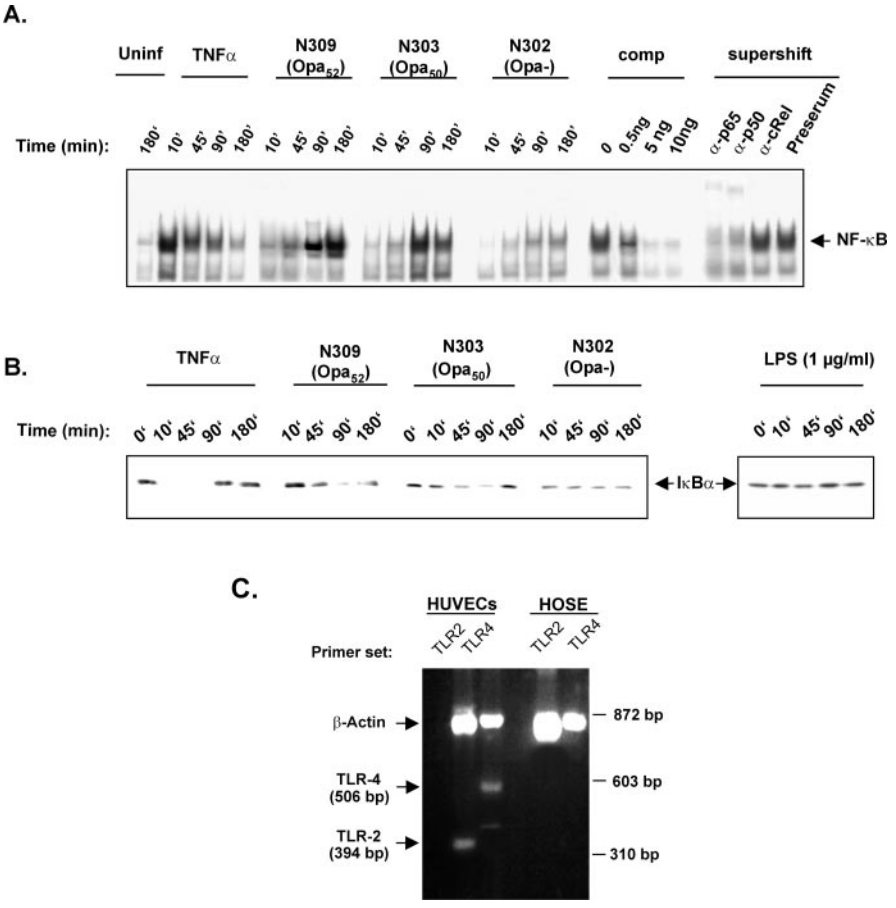

FIG. 7. ***N. gonorrhoeae* infection activates the transcription factor NF-κB.** HOSE cells were either infected with different gonococcal strains, stimulated with 10 ng/ml TNFα, or left untreated. At the indicated periods of time, cells were harvested and fractionated to obtain the cytosolic fraction and the high salt extract of nuclei, as outlined under "Materials and Methods." *A*, the nuclear extracts were incubated with a radioactive labeled DNA fragment (Igκ), which contains the NF-κB binding site, and then subjected to native polyacrylamide gel electrophoresis and autoradiography. The specificity of NF-κB DNA complex formation was investigated by competition with the indicated amounts of unlabeled oligonucleotide (*Comp*). The composition of the induced NF-κB complex was investigated by antibody supershifts using anti-p50, anti-p65, and anti-c-Rel antisera or control preimmune serum (*Preserum*). The position of the protein-DNA complexes is indicated. The data are representative of at least three independent experiments. *B*, cytosolic fractions were prepared at different time points after infection or stimulation with TNFα. Untreated HOSE cells (*Uninf*) were used as a control. The samples were then analyzed in a Western blot using an IκB-specific antibody. HOSE cells were stimulated by the exposure to 1 μg/ml purified LPS. Cytosolic fractions of the same samples were also probed for IκBα to determine the rate of its degradation in response to LPS treatment. The data are representative of at least three independent experiments. *C*, expression of TLR-2 and TLR-4 in HOSE cells. The expression was assessed by semiquantitative RT-PCR using total RNA extracted from HOSE cells and HUVECs as control. Primers specific for β-actin were included in the reaction mixture as an internal control. HUVECs were used as an independent source for human TLR mRNA expression to confirm the previously reported expression of TLR-2 and TLR-4 in these cells (41). The PCR products obtained are indicated with *arrows*. The results are representative of at least three independent experiments.

dition of the ^{32}P-labeled oligonucleotide containing the κB sequence. The reduced mobility of bound oligonucleotide and the supershifts in the presence of anti-p50 and anti-p65 antibodies indicate that these subunits represent the predominant protein species in the κB DNA-binding complex, which becomes activated by gonococcal infection. The time course of active NF-κB appearing in the nuclear fraction following each of these stimuli correlated well with the degradation of IκBα in the cytosol (Fig. 7*B*). Bacterial LPS did not induce IκBα degradation in HOSE cells (Fig. 7*B*). In a number of different cell types, the toll-like receptor-4 (TLR-4) mediates activation of NF-κB in response to LPS. Thus, the finding that LPS did not induce NF-κB in HOSE cells correlates well with our observation that these cells do not express TLR-2 and TLR-4. In contrast to HOSE cells, HUVECs express TLR-4 (Fig. 7*C*), which allows NF-κB activation in response to LPS.

To test whether CEACAM1 expression is directly controlled by NF-κB, we tested whether various inhibitors of NF-κB influence CEACAM1 expression following HOSE stimulation with TNFα or gonococcal infection. We observed an inhibition of CEACAM1 expression when the cells were pretreated with an inhibitory peptide (NF-κB SN50) that inhibits the nuclear translocation of the activated NF-κB complex (Fig. 8). The fact that the NF-κB-specific peptide inhibits CEACAM1 expression clearly indicates that NF-κB is involved in the control of CEACAM1 expression.

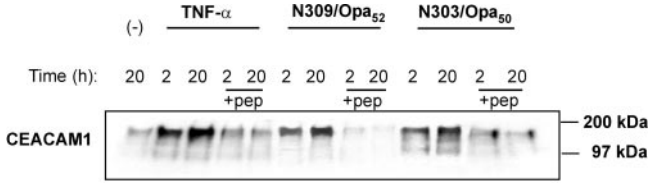

FIG. 8. ***N. gonorrhoeae*-induced expression of CEACAM1 is blocked by inhibitors of NF-κB activation.** HOSE cells were infected with *N. gonorrhoeae* or stimulated using TNFα in the presence or absence of the cell-permeable, inhibitory peptide SN50, which contains the nuclear localization signal sequence of the NF-κB p50 subunit, as indicated. Untreated HUVECs were used as a control. The cell lysates were harvested, and equal amounts of protein were analyzed by Western blot analysis using the CEACAM receptor-specific monoclonal antibody D14HD11. The data are representative of at least three independent experiments.

DISCUSSION

The first critical step in neisserial infection is the interaction with the mucosal epithelium. The Opa-mediated binding to CEACAM receptors might play an important role in this process. Previously, we have examined the mechanism by which *N. gonorrhoeae* stimulates CEACAM1 receptor expression in primary endothelial cells (HUVECs). We provided evidence that LPS from the gonococci induces CEACAM1 expression, which in turn up-regulated bacterial adhesion and thus led to a positive feedback loop with LPS inducing more receptor expression (41). To determine whether this mechanism is not limited to the HUVECs, different epithelial cell lines commonly used as neisserial infection model were analyzed. These cell lines revealed expression of either no or several different CEACAM receptors, which could not be influenced by neisserial infection. This might be due to the fact that CEACAM expression is often strongly dysregulated during malignant transformation (55). The cancer cells used might, therefore, have been unable to respond to signals that would influence CEACAM expression in normal epithelial cells.

In the current study, we therefore characterized the CEACAM expression pattern in HOSE cells that were derived from primary human ovarian epithelium (42). We found significant levels of CEACAM1 and very little CEACAM6 expression. Other members of the CEACAM family were not found in either stimulated or unstimulated cells. CEACAM1 receptor expression was found to be up-regulated during infection with Opa-expressing gonococci, which interacted strongly with HOSE cells, or following stimulation with TNFα (Fig. 1*B*). Interestingly, the nonadherent Opa$^-$ strain N302 induces CEACAM1 expression only late in the infection and to a much lower extent, strongly indicating that bacterial adhesion is required to stimulate CEACAM1 expression (Fig. 5*A*). This result differs from HUVECs, where we could previously show that CEACAM1 up-regulation during gonococcal infection was a contact-independent process.

Immunocytochemical analysis revealed that Opa_{52}-expressing gonococci cause a strong recruitment of CEACAM1 receptors, resulting in a strong co-localization of bacteria with CEACAM1 molecules. In contrast, the heparan sulfate proteoglycan-specific Opa_{50}-expressing bacteria adhered to HOSE cells independently of their CEACAM1 expression level and generally failed to recruit CEACAM1 receptors (Fig. 2). The prolonged exposure of HOSE cells to gonococci resulted in an increased level of Opa_{52}-dependent bacterial binding (Fig. 3*A*). Pretreatment with polyclonal anti-CEACAM antibody prior to infection demonstrated that the increased binding was due to interactions with CEACAM1 receptor(s), because this treatment almost completely blocked interaction of Opa_{52}-expressing gonococci with the HOSE cells (Fig. 3*C*). Consistent with these results, HOSE cells pretreated with TNFα resulted in a significantly increased binding of Opa_{52}-expressing gonococci that was most readily observed early, within 30 min of infection (Fig. 4) and that was sensitive to inhibition by specific immunoglobulins to CEACAM1. These data clearly show that the *N. gonorrhoeae*-induced up-regulation of the CEACAM1 receptor in HOSE cells leads to enhanced adhesion. Since our data show that secretion of endogenous TNFα after neisseral infection was very low even after 24 h, we exclude the possibility that CEACAM1 receptor expression is induced by an autocrine loop involving TNFα.

Opa-expressing gonococci regulate CEACAM1 expression in HOSE cells through the activation of NF-κB (Fig. 7). Supershift experiments demonstrated that the active NF-κB complex consists of a heterodimer comprising the p50 and p65 subunits (Fig. 7*A*), and the inactivation of NF-κB by the specific inhibitory peptide blocked the expression of CEACAM1 (Fig. 8). *N. gonorrhoeae* and TNFα induce CEACAM1 receptor up-regulation in a direct activation process involving NF-κB regulation. Indirect activation of CEACAM1 receptor expression has been described in interferon-γ-stimulated colon cancer cell lines (56). Here, interferon-γ activates interferon regulatory factor-1, which subsequently induces CEACAM1 expression.

We have shown previously that in primary endothelial cells up-regulation of CEACAM1 by *N. gonorrhoeae* is independent of direct contact between bacteria and cells. This process is mediated by bacterial LPS, which activates toll-like receptor-4 (TLR-4) and thereby NF-κB. *N. gonorrhoeae* actively releases large amounts of membrane "blebs," which consist of both protein and lipid components of the outer membrane. Bacterial LPS did not induce IkBα degradation in HOSE cells (Fig. 7*B*). This result correlated well with our finding that TLR-4 was not expressed in HOSE cells (Fig. 7*C*). Furthermore, activation of NF-κB and the subsequent increase of CEACAM1 receptor expression during gonococcal infection seems to be a cell contact-dependent process, since NF-κB activation by the Opa-negative strain was very weak (compare Figs. 5*A* and 7*A*). These data provide evidence that *N. gonorrhoeae* contains components other than LPS that can elicit biological responses via alternative pathways independent of TLR-4. Recent studies have shown that a wide variety of bacterial products, other than LPS and superantigens, can trigger inflammation. It was reported that mammalian TLR-5 recognizes bacterial flagellin from both Gram-positive and Gram-negative bacteria and that activation of the receptor mobilizes NF-κB and stimulates TNFα production (57). For *Pseudomonas* spp., several different factors have been implicated in inducing NF-κB-dependent IL-8 expression, including the binding of pilin to asialo-GM1 receptors on epithelal cells (58) or the secretion of homoserine lactone derivatives (58). Recently, it was shown that cellular responses to bacterial DNA were mediated by TLR-9 (59).

N. gonorrhoeae triggers an NF-κB-dependent up-regulation of CEACAM1 expression in both endothelial and epithelial cells. While the mechanisms and stimuli involved seem to differ between cell types, the result is invariably to increase adherence of bacteria to their target cells by CEACAM-binding Opa variants (Ref. 41; this work). During natural infections the expression of Opa variants and other gonococcal virulence factors is subject to frequent phase variation, and new gonococcal phenotypes expressing functionally distinct combinations of virulence factors constantly arise. These are believed to allow gonococci to colonize diverse human tissues and to persist in a changing environment. Our data identify the expression level of CEACAM1 as one of the factors that may change with time in the naturally infected mucosal surface. If present *in vivo*, the up-regulation of CEACAM1 either by adhering bacteria or by proinflammatory cytokines such as TNFα would probably favor colonization and invasion by CEACAM-binding phenotypic variants. Furthermore, the direct activation by adherent *Neisseria* of NF-κB in mucosal epithelia could be of eminent importance for the innate immune response during neisserial infections by inducing the expression of proinflammatory cytokines/chemokines in addition to CEACAM1.

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Nuclear Factor-κB Directs Carcinoembryonic Antigen-related Cellular Adhesion Molecule 1 Receptor Expression in *Neisseria gonorrhoeae*-infected Epithelial Cells

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