THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 26, Issue of June 29, pp. 24331–24340, 2001

Printed in U.S.A.

Pathogenic *Neisseria* Trigger Expression of Their Carcinoembryonic Antigen-related Cellular Adhesion Molecule 1 (CEACAM1; Previously CD66a) Receptor on Primary Endothelial Cells by Activating the Immediate Early Response Transcription Factor, Nuclear Factor-κB*

Received for publication, August 1, 2000, and in revised form, February 15, 2001
Published, JBC Papers in Press, April 16, 2001, DOI 10.1074/jbc.M006883200

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Neisseria gonorrhoeae **express opacity-associated (Opa) protein adhesins that mediate binding to various members of the carcinoembryonic antigen-related cellular adhesion molecule (CEACAM; previously CD66) receptor family. Although human umbilical vein endothelial cells express little CEACAM receptor *in vitro*, we found neisserial infection to induce expression of CEACAM1, CEACAM1-3L, and CECAM1-4L splice variants. This mediates an increased Opa_{52}-dependent binding of gonococci by these cells. The induced receptor expression did not require bacterial Opa expression, but it was more rapid with adherent bacteria. Because the time course of induction was similar to that seen for induced proinflammatory cytokines, we tested whether CEACAM1 expression could be controlled by a similar mechanism. Gonococcal infection activated a nuclear factor-κB (NF-κB) heterodimer consisting of p50 and p65, and inhibitors that prevent the nuclear translocation of activated NF-κB complex inhibited CEACAM1 transcript expression. Each of these effects could be mimicked by using culture filtrates or purified lipopolysaccharide instead of intact bacteria. Together, our results support a model whereby the outer membrane "blebs" that are actively released by gonococci trigger a Toll-like receptor-4-dependent activation of NF-κB, which up-regulates the expression of CEACAM1 to allow Opa_{52}-mediated neisserial binding. The regulation of CEACAM1 expression by NF-κB also implies a broader role for this receptor in the general inflammatory response to infection.**

The important human pathogens *Neisseria gonorrhoeae* and *Neisseria meningitidis* possess the ability to colonize human mucosal tissue and then penetrate into deeper tissues to cause invasive disease. Initial contact with host tissues is thought to be mediated by neisserial type IV pili, and a tight secondary interaction can then be established by the bacteria's phase-variable, colony opacity-associated (Opa)[1] outer membrane proteins. There are two distinct classes of Opa proteins based on their differential binding specificity for cellular receptors. One class targets the *Neisseriae* to cell surface heparan sulfate proteoglycan (HSPG) receptors (1, 2) and, via binding to the extracellular matrix proteins vitronectin and fibronectin, to cell surface integrins (3, 4). Other Opa proteins bind to the CD66 epitope-containing members of the *c*arcino*e*mbryonic *a*ntigen-related *c*ellular *a*dhesion *m*olecules (CEACAM), which are expressed differentially on multiple tissues throughout the human host (5, 6). Some Opa proteins interact with both HSPG and CEACAM receptors (7, 8), presumably via distinct binding sites; however, each variant appears to be able to mediate host cellular invasion only via either one or the other receptor class (9).

CEACAM1 (previously called BGP or CD66a; new nomenclature for this and other CEA family members was introduced by Beauchemin *et al.* (42)), CEACAM3 (CGM1 or CD66d), CEACAM5 (CEA or CD66e), and CEACAM6 (NCA or CD66c) can all serve as receptors for the pathogenic *Neisseria* spp.; however, individual Opa variants are specific for various combinations of these closely related proteins (7, 8, 10–15). The closely related receptors CEACAM4, CEACAM7, and CEACAM8 are not bound by any Opa variants tested to date (16). Each CEACAM receptor consists of an immunoglobulin variable domain-like region followed by up to six immunoglobulin constant domain-like structures (6). CEACAM1 and CEACAM3 are inserted into the cellular membrane via a carboxyl-terminal transmembrane and cytoplasmic domain, whereas CEACAM5, CEACAM6, CEACAM7, and CEACAM8 possess glycosylphosphatidylinositol anchors. Even though 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 (17). These different binding specificities may have important

* This study was supported in part by Deutsche Forschungsgemeinschaft Grants Me705/5-1 and Na292/5-2 and by the Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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** Supported by Medical Research Council of Canada Grant MT-15499.

[1] The abbreviations used are: Opa, opacity-associated; HSPG, heparan sulfate proteoglycan; CEACAM, carcinoembryonic antigen-related cellular adhesion molecule; HUVECs, human umbilical vein endothelial cells; TNF-α, tumor necrosis factor α; NF-κB, nuclear factor κB; HDMECs, human dermal microvascular endothelial cells; LPS, lipopolysaccharide; IκBα, inhibitory protein κBα; TPCK, tosylphenylalanyl chloromethyl ketone; PSI, proteasome inhibitor; RT-PCR, reverse transcription-polymerase chain reaction; bp, base pairs; TLR, Toll-like receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL, interleukin.

implications for the pathogenic process of *Neisseria* because 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 (6, 18–21), suggesting that the cellular response to neisserial binding depends upon the specific combination of CEACAM receptors engaged.

Even though CEACAM receptors are part of the immunoglobulin superfamily, their functions are poorly understood. They mediate intercellular adhesion via both homotypic (CEACAM1, CEACAM5, and CEACAM6) and/or heterotypic (CEACAM5-CEACAM6 and CEACAM6-CEACAM8) interactions (22, 23). 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 because 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*, and this effect was abrogated by deleting the receptor's cytoplasmic domain (24, 25). Such a growth-inhibitory role is consistent with clinical observations that CEACAM1 expression is down-regulated in various colonic carcinomas (26, 27). Together, these features imply a role for members of the CEACAM receptor family as sensory and regulatory molecules in cell-cell adhesion events (28).

CEACAM1, CEACAM3, and CEACAM6 are expressed by human polymorphonuclear neutrophils and can mediate gonococcal binding and opsonin-independent phagocytosis by these phagocytes (10, 11, 14, 29). This interaction appears to play a central role in the pathogenic process because a urethral exudate consisting primarily of polymorphonuclear neutrophils associated with both extracellular and intracellular 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, CEACAM5, and CEACAM6 on their apical surface, and Opa binding to these receptors mediates bacterial uptake, cellular transcytosis, and release at the basolateral surface (30). 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 (31). Because both of these pathogens can also cause invasive disease, interactions with the endothelia must also occur. Primary human umbilical vein endothelial cells (HUVECs) grown in culture express little CEACAM receptor. There is, however, a substantial up-regulation of CEACAM1 expression after treatment with the proinflammatory cytokine tumor necrosis factor α (TNF-α) (13, 15, 32), which is present in serum at high levels during invasive meningococcal disease (33–35). This increased up-regulation of CEACAM1 correlates with an increased adherence and invasion of different Opa-expressing bacteria into these cells *in vitro* (13, 15). In the present study, we observed that prolonged infection of HUVECs resulted in an increased binding of gonococci expressing the CEACAM receptor-specific Opa$_{52}$ adhesin. We have shown previously that *N. gonorrhoeae* infection induces TNF-α expression by epithelial cells (36). If a similar response occurs during neisserial infection of endothelial cells, then these bacteria could presumably induce an autocrine loop that results in the expression of CEACAM1. We thus determined the kinetics of CEACAM1 expression pattern after neisserial infection and related these to the induced cytokine profile. We demonstrate that the *CEACAM1-4L* and *CECAM1-3L* splice variants are both induced with a time course similar to that of proinflammatory cytokines, including TNF-α. Our results indicate that the activation of nuclear factor kappa B (NF-κB) directly triggers CEACAM1 expression and mediates increased Opa-dependent bacterial binding to HUVECs. This phenomenon could clearly contribute to neisserial attachment to and penetration into the vasculature during invasive disease. It also has broad implications for our understanding of the natural role of CEACAM1 because its regulation by the immune regulator factor NF-κB implies a role in the innate response of endothelial cells to infection. This represents the first example that we are aware of in which a human pathogen directly induces the expression of its receptor by a target host cell.

MATERIALS AND METHODS

Cell Lines—HUVECs were obtained from human umbilical vein by chymotrypsin digestion as described previously (37) and then grown in low serum endothelial cell growth medium (PromoCell, Heidelberg, Germany) using flasks precoated with 0.2% gelatin in a humidified atmosphere at 37 °C with 5% CO_2. HUVECs at passage 4 were grown to form a confluent monolayer and then seeded to new precoated flasks or into wells containing gelatin-coated glass coverslips to obtain a confluence of about 60%. Human dermal microvascular endothelial cells (HDMECs) were cultured in MCDB-131 medium (Life Technologies, Inc.) with 10% heat-inactivated fetal calf serum and used between passages 4 and 5.

Bacterial Strains—Construction of the recombinant strains invariantly synthesizing the 11 genetically defined Opa proteins of *N. gonorrhoeae* MS11 were described previously by Kupsch *et al.* (9). 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. Daily subculture of all strains was carried out using a binocular microscope to select for desired Opa phenotypes. Opa protein expression patterns were confirmed by SDS-polyacrylamide gel electrophoresis and immunoblot analysis of total bacterial extracts using the monoclonal antibody 4B12C11 (38), which was generously provided by Dr. Mark Achtman (Berlin, Germany). Recombinant *Escherichia coli* strains expressing *N. meningitidis* Opa variants were also described previously (15).

Bacterial Infection Assays and Stimulation of HUVECs—For infection experiments, HUVECs 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 (Life Technologies, Inc.) supplemented with 10% fetal calf serum. Gonococci were harvested from fresh overnight cultures into M199 medium containing 10% heat-inactivated fetal calf serum to obtain a culture density of 10^8 colony-forming units/ml and then used to infect HUVECs at a multiplicity of infection of 10–20 bacteria/cell for the indicated time points. For immunofluorescence analysis, HUVECs were infected as outlined above except that cells were initially seeded onto gelatin-coated 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 the levels of gonococcal adherence and invasion, the gonococci were stained for immunofluorescence and then analyzed by confocal laser scanning microscopy as described previously (15, 30). Where indicated, various other stimuli were added directly to the culture medium. TNF-α was purchased from R&D Systems. Where indicated, purified mouse anti-human TNF-α monoclonal antibodies (BD PharMingen, San Diego) were added at 15 μg/ml just prior to infection. The polyclonal anti-human CEACAM antiserum was puchased from DAKO (Glostrup, Denmark). Experiments involving LPS were done using LPS prepared from *E. coli* serotype O111:B4 and/or *Salmonella typhimurium* by phenol extraction (Sigma). LPS suspensions were sonicated in endotoxin-free water (Life Technologies, Inc.) to disperse any aggregates formed and were then diluted to the indicated final concentration in supplemented medium. Neisserial LPS was purified according to the method of Galanos *et al.* (62) and was generously provided by Dr. Anne Muller from our group (Berlin).

Immunoblotting—CEACAM1 protein expression in response to exposure to bacterial strains, TNF-α, 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

protein were separated by SDS-polyacrylamide gel electrophoresis (10–11%) and blotted onto Immobilon P transfer membranes (Millipore). Western blot analysis was performed using the CEACAM1, CEACAM3, CEACAM5, and CEACAM6 cross-specific monoclonal antibody D14HD11, the CEACAM6-specific antibody 9A6 (Immunotech, Marseille, France), and the CEACAM1 and CEACAM5 cross-specific antibody 4/3/17. D14HD11 and 4/3/17 were both generously provided by Dr. Fritz Grunert, University of Freiburg, Germany. Bound antibodies were detected using a peroxidase-conjugated goat anti-mouse secondary antibody and the ECL chemiluminescent detection system (Amersham Pharmacia Biotech).

To test for IκBα degradation, cytosolic fractions obtained from HUVECs exposed to various stimuli were analyzed by immunoblot analysis using an IκBα-specific polyclonal antibody that does not cross-reactive with other IκB family members (C-21; Santa Cruz Biotechnology).

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. Cells were pretreated with 50 μg/ml of this peptide for 15 min at 37 °C before TNF-α or the bacteria were added. To confirm the role of NF-κB in CEACAM1 expression, two other inhibitors with different mechanisms of action were also used. The cells were pretreated either with the serine protease inhibitor tosylphenylalanyl chloromethyl ketone (TPCK; Sigma) or a proteasome inhibitor (PSI) obtained from Calbiochem-Novabiochem Ltd. (U. K.) for 30 min before addition of the stimuli.

To inhibit phosphorylation-dependent steps involved in the activation of NF-κB, HUVECs seeded in 75-cm^2 flasks were either not pretreated or were pretreated with 1 μM herbimycin A for 24 h or 100 mM genistein for 1 h and then either infected with N309 or stimulated with TNF-α. The effects of these inhibitors on CEACAM1 expression levels were then determined as indicated.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Analysis—Total RNA was isolated from HUVECs that had been treated with various stimuli, as indicated, using either Trizol reagent (Life Technologies, Inc.) 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 (Life Technologies, Inc.) and oligo(dT) primers. As a control, the synthesis of cDNA was performed in the absence of reverse transcriptase. 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 (Life Technologies, Inc.) for 33 cycles with an annealing temperature of 56 °C. The primers used were 5′-primer B1 (ACAGTCAAGACGATCATAGT) and 3′-primer C2 (ATCTTGTTAGGTGGGTCATT), resulting in amplified fragments of between 189 and 530 bp (39). Amplification of cytokine DNA was done using the primers that we have described previously (36). To detect Toll-like receptor (TLR) 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 (40). Depending on which primer sets were used for the primary amplification, primers specific for the constitutively expressed housekeeping gene GAPDH or β-actin was also included within the reaction mixture to provide an internal control that allowed samples to be loaded equally. In each case, PCR products were visualized by ethidium bromide staining after agarose gel electrophoresis.

Electrophoretic Mobility Shift Assay—At the indicated time points after infection, cytoplasmic and nuclear extracts were prepared by using the non-ionic detergent method described previously (36). Gel retardation assays for the detection of active NF-κB complex were performed using an Igκ oligonucleotide that had been labeled using the large fragment DNA polymerase (Klenow) in the presence of deoxy-[α-^{32}P]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 described previously (36). 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, and the gels were then dried and exposed to Amersham TM films (Amersham Pharmacia Biotech) at −70 °C using an intensifying screen.

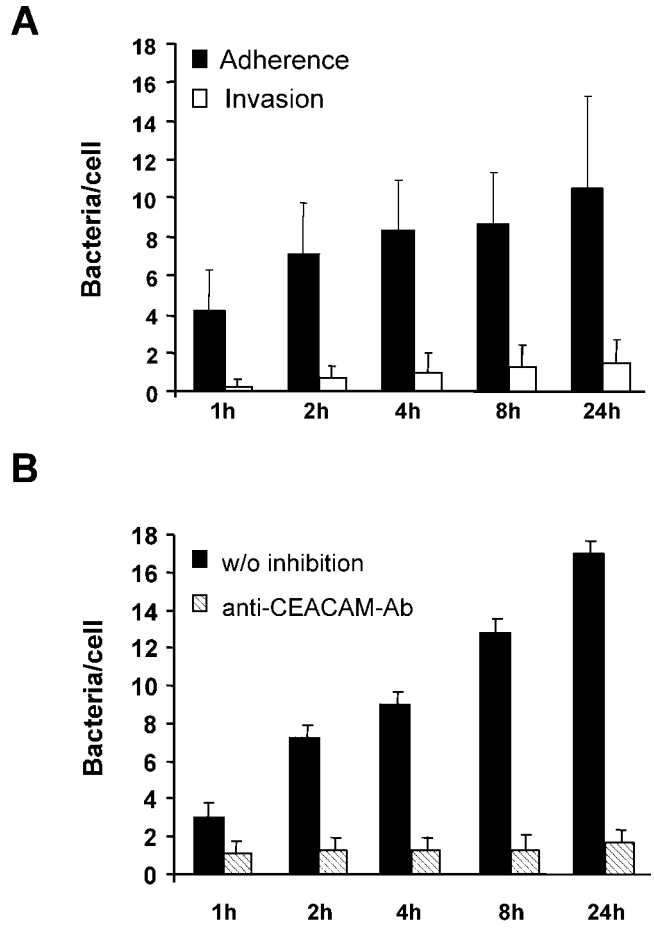

FIG. 1. **CEACAM-dependent association of *N. gonorrhoeae* with primary endothelial cells increases with time.** HUVECs seeded on glass coverslips were infected with N309/Opa$_{52}$ over a time period from 1 to 24 h. At the indicated time points, cells were fixed and then labeled immunofluorescently for analysis by confocal laser scanning microscopy. *Panel A*, total associated and intracellular bacteria/cell were counted. *Black bars* show adherence to untreated HUVECs; *open bars* show intracellular bacteria associated with untreated HUVECs. *Panel B*, HUVECs seeded on glass coverslips were pretreated with 100 μg/ml anti-CEACAM antibody (*Ab*) for 1 h (*cross-hatched bars*) or left untreated (*black bars*). HUVECs were then infected with N309/Opa$_{52}$ over a time period from 1 to 24 h, and total associated bacteria/cell is shown. Assays were performed in triplicate on at least three separate occasions, and data illustrate the mean ± S.D. of one representative experiment.

RESULTS

Neisserial Infection Induces CEACAM1 Expression by Endothelial Cells—Previously, we had found very little CEACAM1 receptor expression by HUVECs unless they were prestimulated with the proinflammatory cytokine TNF-α. Other members of the CEA receptor family were not found in either unstimulated or stimulated HUVECs. Consistent with this, *N. gonorrhoeae* or recombinant *E. coli* expressing either gonococcal or meningococcal CEACAM-specific Opa proteins showed only low levels of binding to HUVECs using standard (*i.e.* 3 h) *in vitro* infection assays unless the HUVECs were pretreated with TNF-α (13, 15). However, we found that extended infection resulted in consistently increasing levels of Opa-mediated bacterial binding to otherwise unstimulated HUVECs, and this correlated with an increased level of bacterial internalization (Fig. 1*A*). To demonstrate that the increased binding was caused by interactions with CEACAM receptor(s), HUVECs were pretreated with polyclonal anti-CEACAM antibody prior to infection. This treatment blocked interaction with the HUVECs almost completely, and the increased bacteria binding with time was no longer evident (Fig. 1*B*).

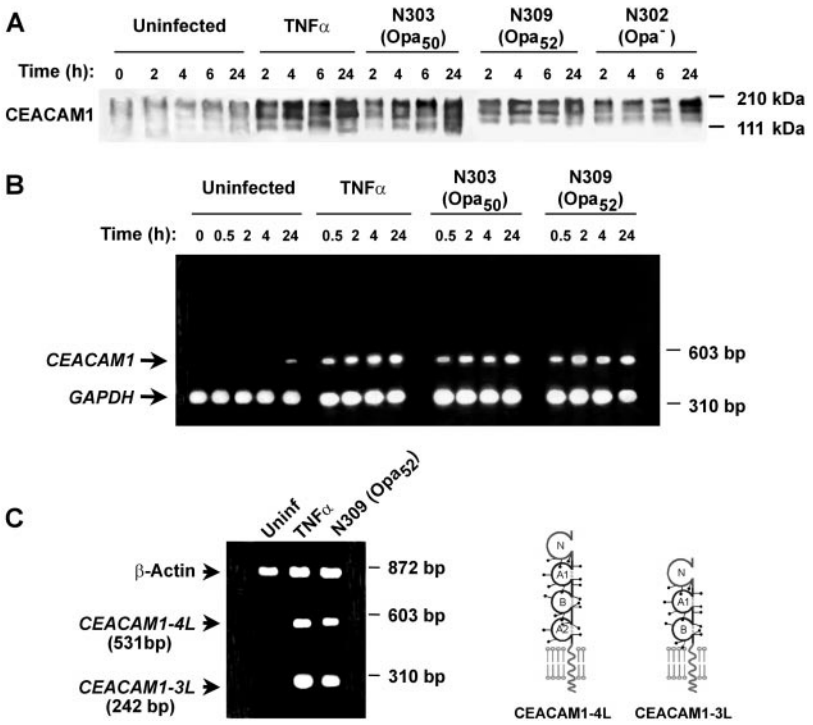

FIG. 2. **Effect of *N. gonorrhoeae* infection on CEACAM expression by HUVECs.** *Panel A*, HUVECs were either left untreated, infected with different gonococcal strains (N303/Opa_{50}, N309/Opa_{52}, N302/Opa^-), or stimulated with 10 ng/ml TNF-α. Total protein was isolated at the indicated time points, separated by SDS-polyacrylamide gel electrophoresis, and immunoblots were probed with the CEACAM receptor-specific monoclonal antibody D14HD11. *Panel B*, *CEACAM1* transcript expression by HUVECs. Total RNA was isolated after the indicated time intervals following the addition of TNF-α 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, the transcript encoding the housekeeping protein GAPDH was coamplified as an internal control to assure that equal samples were applied. *Panel C*, expression of the CEACAM1–4L (BGPa) and CECAM1-3L (BGPb) splice variants by HUVECs. HUVECs were either left untreated, infected with gonococcal strain N309/Opa_{52}, or stimulated with 10 ng/ml TNF-α. 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 coamplification 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. The schematic drawings that illustrate the domain structure of CEACAM1–4L and -3L are used here with the permission of Fritz Grunert (University of Freiburg, Germany).

To determine whether these increased interactions could result from increased CEACAM receptor expression, we performed immunoblot analysis of cellular lysates prepared at various intervals after infection. The HUVECs were either left untreated, infected with various gonococcal strains, or stimulated with TNF-α as a positive control. Expression of the CEACAM1 protein was found to be induced during neisserial infection, and the time course of this induction was similar to that seen when stimulating the cells with purified TNF-α (Fig. 2*A*). No other CEACAM family member(s) were detected in either stimulated or unstimulated cells. The rapid induction of CEACAM1 expression after infection was also confirmed by FACS analysis (data not shown) and by semiquantitative RT-PCR to detect CEACAM1-encoding transcript (Fig. 2*B*). Importantly, *N. gonorrhoeae* strains expressing either the HSPG receptor-specific Opa_{50} or the CEACAM receptor-specific Opa_{52}, and the nonadherent Opa^- strain N302 induced CEACAM1 expression (Fig. 2, *A* and *B*). This implies that this effect was not likely the result of a specific signal directly downstream of Opa binding to one of its cellular receptors. We generally observed increased levels of three defined protein bands by immunoblot analysis using the CEACAM receptor-specific monoclonal antibody D14HD11 (Fig. 2*A*) and have confirmed that all three bands represent the CEACAM1 receptor by comparing blots probed with various CEACAM-specific antibodies (data not shown). This banding pattern likely results from a combination of the variable glycosylation of CEACAM1 and/or the expression of multiple splice variants because the relative levels of each of these can vary among cells and cell lines (41). 13 different CEACAM1 splice variants are known to exist. To analyze which splice variant(s) are induced in endothelial cells, we performed RT-PCR experiments with RNA from unstimulated, TNF-α-treated, and gonococcal-infected HUVECs. 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 discriminate among known splice variants according to the size of the RT-PCR products (39). Stimulated endothelial cells were found to express only 2 of the 13 known splice variants, as shown in Fig. 2*C*. The larger RT-PCR product revealed a size of 531 bp, which corresponds to CEACAM1-4L (BGPa) expression, whereas the second RT-PCR product (242 bp) indicates that the CEACAM1-3L (BGPb) splice variant which lacks the A2 domain, is also expressed. Both splice variants were expressed in equal amounts (Fig. 2*C*), and each 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 (42). The same pattern of splice variants was observed whether the HUVECs were treated with TNF-α (Fig. 2C) or infected with *N. gonorrhoeae* N303/Opa_{50} (data not shown).

We have shown previously that TNF-α expression by epithelial cells is induced by gonococcal infection (36). If this also happens in HUVECs, then an autocrine loop involving *de novo* TNF-α expression which leads to the subsequent induction of CEACAM1 expression could presumably explain the increased gonococcal binding seen in Fig. 1. To assess changes in cytokine gene expression in response to gonococcal infection of endothelial cells, we used specific primers to perform semiquantitative

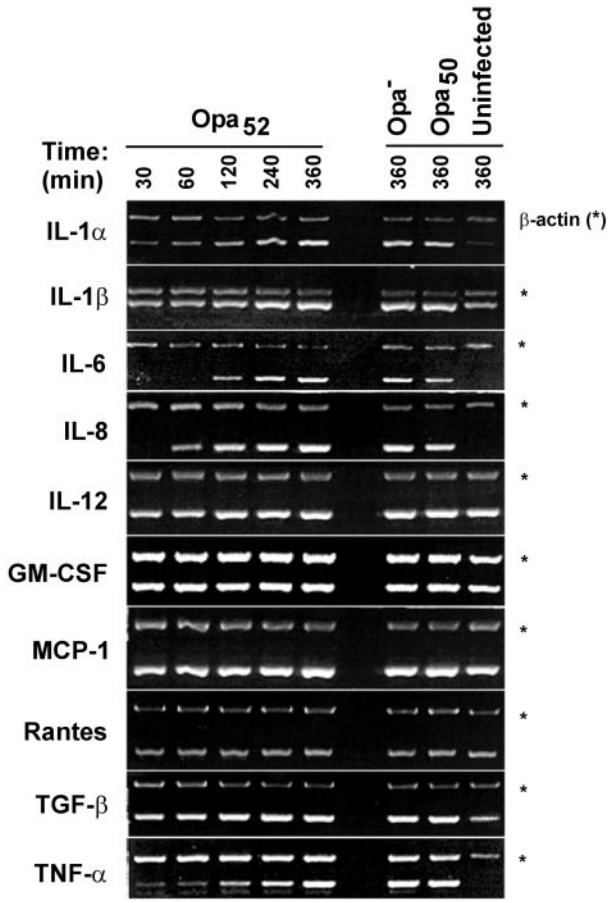

FIG. 3. **Analysis of cytokine mRNA levels in HUVECs in response to *N. gonorrhoeae* infection.** Total RNA isolated from HUVECs that had either been left untreated or were infected by different gonococcal strains for the indicated times was analyzed by RT-PCR using cytokine-specific primer pairs, as indicated. β-Actin mRNA primers were included in each reaction as an internal control to assure equal loading, and the amplified β-actin transcript is indicated (*). Similar results were obtained in more than three independent experiments. *GM-CSF*, granulocyte-macrophage colony-stimulating factor; *MCP-1*, monocyte chemoattractant protein 1; *TGF-β*, transforming growth factor β; *Rantes*, regulated on activation normal T cell expressed and secreted.

RT-PCR from total RNA isolated at various time points after infection (Fig. 3). *N. gonorrhoeae* strains expressing either no Opa (N302), Opa_{52} (N309), or Opa_{50} (N303) induced the expression of several cytokines, including IL-1α, IL-6, IL-8, and TNF-α, whereas the expression levels of other cytokines did not change. Because CEACAM1 expression was induced as quickly as any of these cytokines (*i.e.* compare Figs. 2*B* and 3), it is unlikely that newly expressed cytokines are responsible for the induced CEACAM1 receptor expression. However, because we have shown previously that TNF-α stimulates CEACAM1 expression (13, 15), we determined that induced TNF-α was required for CEACAM1 expression by using antibodies that inhibit the activity of TNF-α. These antibodies clearly reduced CEACAM1 protein expression in response to TNF-α treatment but not in response to neisserial infection (Fig. 4), thus confirming that this cytokine is not essential for the induced receptor expression.

Neisserial Infection Activates NF-κB in Endothelial Cells—Proinflammatory cytokines are controlled by the immediate early transcription factor NF-κB. NF-κB is normally found in the cytoplasm as a complex with IκB, which blocks NF-κB translocation to the nucleus and thus prevents transcription of NF-kB regulated genes. Upon cellular activation, IκB dissociates from NF-κB and is degraded by the proteasome, thereby allowing translocation of the released NF-κB into the nucleus. Subconfluent monolayers of HUVECs were treated with TNF-α or infected with *N. gonorrhoeae* expressing either the HSPG-specific Opa_{50} or the CEACAM-specific Opa_{52}. At different time points after challenge, the cells were harvested and fractionated to separate the cytosol from the nuclei. The resulting nuclear protein extracts were then analyzed for the levels of DNA binding activity using an electrophoretic mobility shift assay with a radioactively labeled oligonucleotide corresponding to the DNA binding site of NF-κB (Fig. 5*A*). Nuclear protein binding of the oligonucleotide was observed within 10 min of infection by N303/Opa_{50}, and this increased until 90 min postinfection before declining again by 180 min. N309/Opa_{52} also induced NF-κB activation, although this happened after a short delay (*i.e.* by 45 min). TNF-α treatment of the HUVECs resulted in a more rapid translocation of NF-κB, with strong binding being observed by 10 min postinfection. The time course of active NF-κB appearing in the nuclear fraction following each of these stimuli correlated well with the disappearance of IκBα from in the cytosol (Fig. 5*B*). Consistent with the CEACAM1 protein expression being independent of TNF-α expressed by the infected HUVECs (Fig. 4*A*), anti-TNF-α antibodies clearly reduced IκBα degradation in response to added TNF-α but had little effect on that seen in response to neisserial infection (Fig. 4*B*).

We then performed competition band shift assays using the unlabeled oligonucleotide consensus sequence to confirm the specificity of the binding activity seen, and a decrease in the formation of radioactively labeled complex was observed with increasing concentration of unlabeled NF-κB consensus sequence (Fig. 5*A*). Because several different homodimeric or heterodimeric forms of NF-κB exist, the nature of the activated transcription factor was characterized using supershift assays (Fig. 5*A*). Experiments were performed in which the nuclear extracts were preincubated with anti-p50, anti-p65, anti-c-Rel, or preimmune serum before addition of the ^{32}P-labeled oligonucleotide containing the κB sequence. The reduced mobility of bound oligonucleotide in the presence of anti-p50 and anti-p65 antibodies indicates that these subunits represent the predominant protein species in the κB DNA-binding complex that becomes activated by gonococcal infection.

To determine whether CEACAM1 expression is controlled directly by NF-κB, we tested whether various inhibitors of NF-κB function influenced CEACAM1 expression after HUVEC stimulation with TNF-α and/or gonococcal infection. We observed an inhibition of CEACAM1 transcript expression when the cells were pretreated with either the serine protease inhibitor TPCK or a PSI that inhibits the 20 S proteasome (Fig. 6*B*) and a corresponding reduction in CEACAM1 protein seen by Western blot (Fig. 6*A*). An inhibitory effect was also seen by Western blot (Fig. 6*C*) and semiquantitative RT-PCR (data not shown) when the cells were pretreated with an inhibitory peptide (NF-κB SN50) that inhibits the nuclear translocation of activated NF-kB complex. Consistent with this, NF-κB SN50 reduced the apparent increase in CEACAM-dependent bacterial binding seen during prolonged infection (Fig. 7). TPCK, PSI, and the NF-κB-specific peptide all inhibit NF-κB induction of transcription by different mechanisms, and the fact that each also inhibits CEACAM1 expression clearly indicates that NF-κB is involved in the control of CEACAM1 expression.

A tyrosine kinase-dependent step has been reported to occur upstream of NF-κB activation in some systems (43, 44) but not in others (45). We therefore tested whether the broadly specific tyrosine kinase inhibitor genistein and/or the Src family kinase-specific inhibitor herbimycin A had an effect on

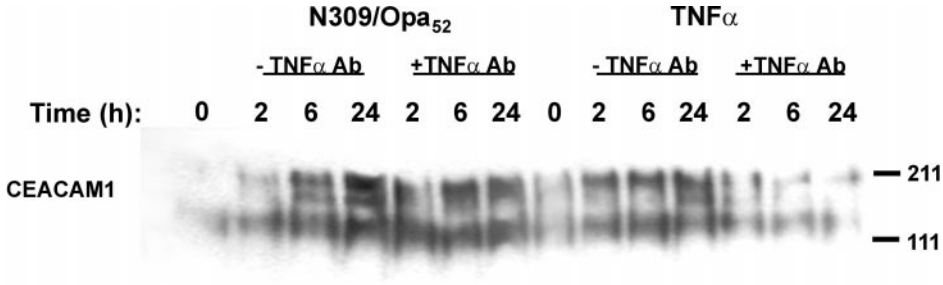

FIG. 4. **TNF-α expressed by infected HUVECs is not required for the induced expression of CEACAM1.** HUVECs were either infected with N309/Opa$_{52}$ or stimulated by the addition of 20 ng/ml TNF-α, each in the presence (+) or absence (−) of 15 μg/ml anti-human TNF-α monoclonal antibody (*Ab*). As a control HUVECs were left untreated. Total protein was isolated at the indicated time points, separated by SDS-polyacrylamide gel electrophoresis, and immunoblots were probed with either the CEACAM receptor-specific monoclonal antibody D14HD11 or IκBα-specific antibody, respectively. Similar results were obtained in more than three independent experiments.

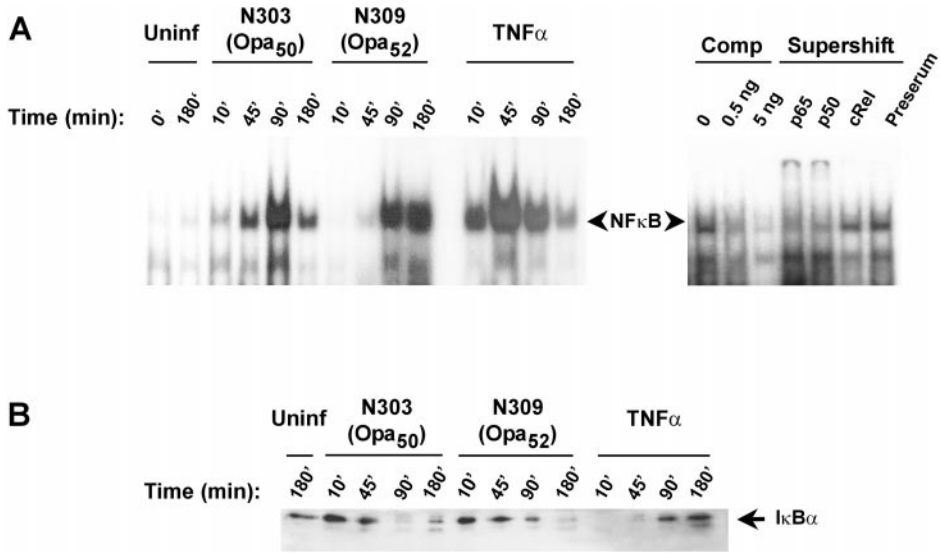

FIG. 5. ***N. gonorrhoeae* infection activates the transcription factor NF-κB.** HUVECs were either infected with various gonococcal strains, stimulated with 10 ng/ml TNF-α, or left untreated (*Uninf*). At the indicated time points, cells were harvested and fractionated to obtain the cytosolic fraction and the high salt extract of nuclei, as described under "Materials and Methods." *Panel A*, the nuclear extracts were incubated with radioactively 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 (*Comp*) with the indicated amounts of unlabeled oligonucleotide. The composition of the induced NF-κB complex was investigated by antibody supershifts using anti-p50, anti-p65, anti-c-Rel antisera or control preimmune serum (*Preserum*). The positions of the protein-DNA complexes are indicated. The data are representative of at least three independent experiments. *Panel B*, cytosolic fractions were prepared from samples taken at different time points after infection or stimulation with TNF-α. Untreated HUVECs were used as a control. The samples were then probed by Western blot analysis using an IκBα-specific antibody.

CEACAM1 expression (Fig. 8). Both inhibitors down-regulated the sustained expression of CEACAM1 transcript after treatment with either TNF-α or gonococci, although low levels of transcript were still detected at early time points (*i.e.* 0.5 and 2 h postinfection) in the presence of these inhibitors.

Characterization of a Neisserial Factor That Activates NF-κB—The finding that CEACAM1 expression was induced by gonococci regardless of the receptor specificity of their expressed Opa (Fig. 2) suggests that NF-κB activation is not a specific response triggered by simple engagement of one of these receptors. The fact that the Opa$^-$ strain N302 also induces CEACAM1 expression (Fig. 2) also implies that bacterial binding to the HUVECs is not necessary. To test whether the factor that activates NF-kB is released into the culture supernatant, we passed cultures of gonococcal strain N303/Opa$_{50}$ through a 0.2-μm filter to remove intact bacteria but not soluble factors released by the gonococci. At various intervals after the addition of filtrate to the HUVECs, total cellular protein was isolated and analyzed. A clear induction of CEACAM1 was seen to follow exposure of HUVECs to gonococcal filtrates (Fig. 9*A*), and there was no obvious difference with respect to the time course of this effect *versus* that seen in response to infection by whole bacteria (*i.e.* compare with Fig. 2*A*).

Gonococci are known to shed large amounts of outer membrane in the form of "blebs" that contain both LPS and outer membrane proteins. LPS is a potent activator of cells of the immune and inflammatory systems, including macrophage, monocytes, and endothelial cells. It is bound by the serum LPS-binding protein, and this complex is then bound by the CD14 receptor (46, 47). Endothelial cells do not express CD14, but instead use soluble CD14 from serum to respond to LPS (48). We thus tested whether purified LPS could itself mediate the induction of CEACAM1 expression. As seen during neisserial infection, purified LPS triggered both the disappearance of IκBα by 1.5 h (data not shown) and the subsequent expression of CEACAM1 by 2.5 h (Fig. 9*B*). We tested various concentrations of LPS that had been purified from various bacterial species with similar results (data not shown). Neither the membrane-bound (mCD14) nor the serum-soluble (sCD14) LPS receptors are themselves competent to induce intracellular signaling or downstream cellular responses to LPS. Recently, it has been reported that the sCD14·LPS-binding protein complex interacts with distinct members of the TLR family. In particular, TLR-4 appears to mediate the activation of NF-κB in re-

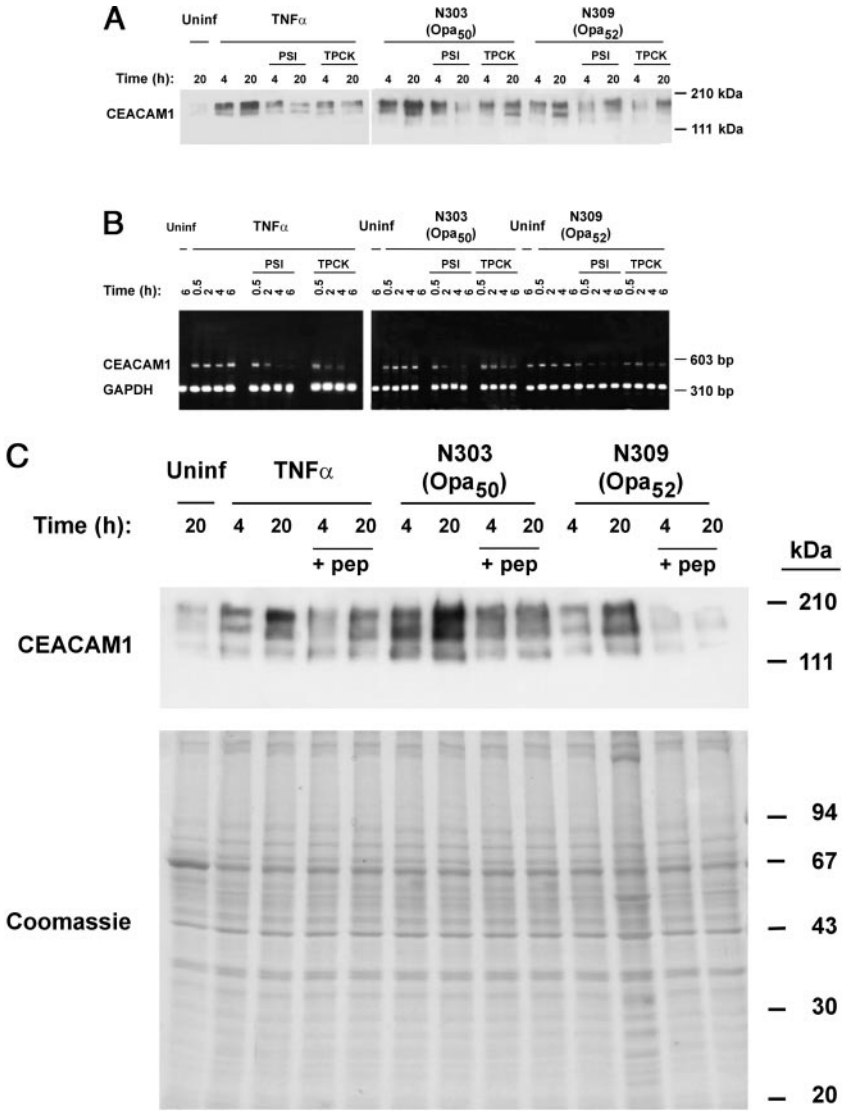

FIG. 6. ***N. gonorrhoeae*-induced expression of CEACAM1 is blocked by inhibitors of NF-κB activation.** *Panel A*, HUVECs were pretreated with 25 μM TPCK or 50 μM PSI for 30 min, as indicated. The cells were subsequently infected with N303/Opa_{50}, N309/Opa_{52}, or treated with 10 ng/ml TNF-α. Untreated HUVECs (*Uninf*) were used as a control. The cell lysates were harvested and analyzed by Western blot analysis using the CEACAM receptor-specific monoclonal antibody D14HD11. *Panel B*, effect of TPCK and PSI on *CEACAM1* transcript expression. Total RNA was isolated from HUVECs at the indicated time points after infection with gonococcal strains or stimulation with TNF-α, and then *CEACAM1* expression levels were assessed by semiquantitative RT-PCR. Expression of the housekeeping gene encoding GAPDH was used as an internal control. *Panel C*, HUVECs were infected with *N. gonorrhoeae* or stimulated using TNF-α in the presence or absence of the cell-permeable inhibitory peptide SN50, which contains the NLS sequence of the NF-κB p50 subunit, as indicated. Untreated HUVECs were used as a control. The cell lysates were harvested and analyzed by Western blot analysis using the CEACAM receptor-specific monoclonal antibody D14HD11. The Coomassie Blue-stained figure was included to confirm that variations in CEACAM1 expression were not caused by differences in sample loading. The data are representative of at least three independent experiments.

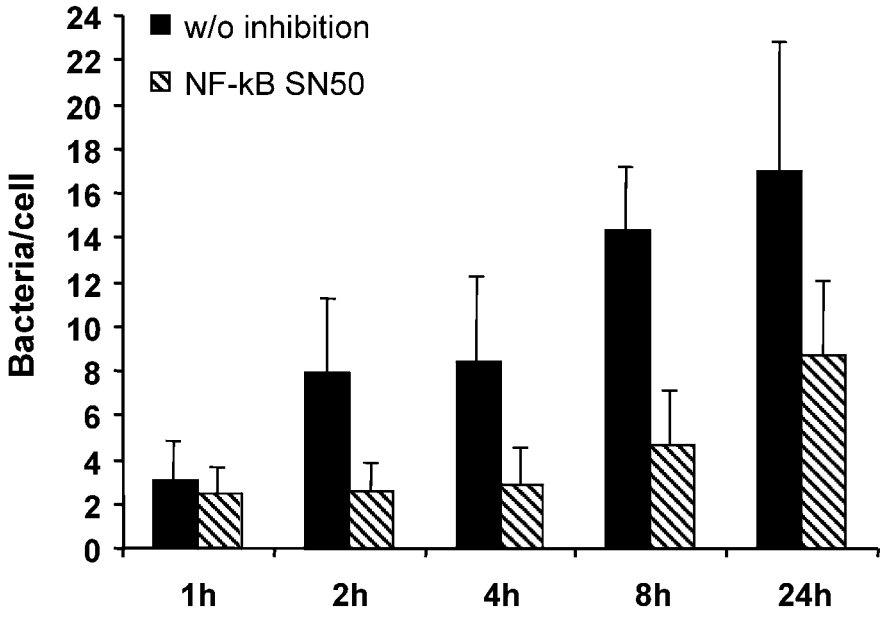

FIG. 7. **NF-kB activity is required for the increased association of *N. gonorrhoeae* with HUVECs over time.** HUVECs seeded onto glass coverslips were treated with 50 μg/ml inhibitory peptide NF-κB SN50 (*cross-hatched bars*) or left untreated (*black bars*) for 30 min prior to infection by N309/Opa_{52}. At the indicated times, samples were fixed and analyzed by confocal laser scanning microscopy to determine total associated bacteria/cell. The untreated control in this figure and in Fig. 1*B* are the same because the anti-CEACAM antibody and the SN50 inhibitor samples were performed in parallel. This assay was performed in triplicate on at least three separate occasions, and data illustrate the mean ± S.D. of one representative experiment.

sponse to LPS (49, 50). The closely related TLR-2 has been shown to induce a similar cellular response in the presence of other cell wall components (55–58, 60). We thus confirmed that our HUVECs expressed these receptors by using semiquantitative RT-PCR. We found TLR-2 and TLR-4 both to be expressed (Fig. 10), however we did not find an obvious difference

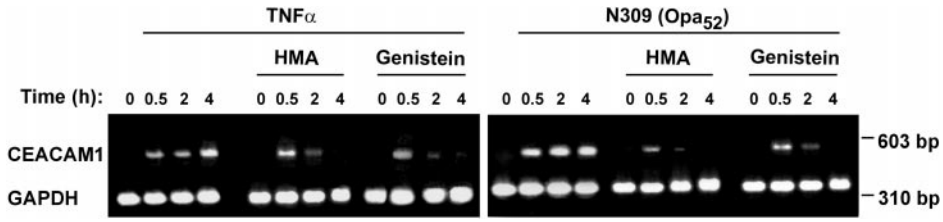

FIG. 8. **Effect of protein tyrosine kinase inhibitors on the expression of *CEACAM1*.** HUVECs were untreated or pretreated with 1 μM herbimycin A (*HMA*) for 24 h or 100 μM genistein for 1 h, infected with N309/Opa$_{52}$, or treated with 10 ng/ml TNF-α. Total RNA was isolated at the indicated time points and analyzed by RT-PCR using GAPDH expression as an internal control. Similar results were obtained in more than three independent experiments.

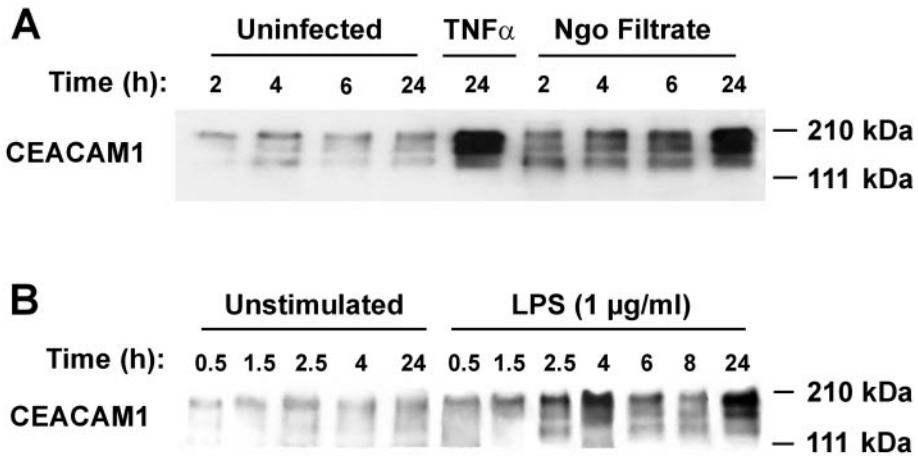

FIG. 9. **Induction of CEACAM1 expression by *N. gonorrhoeae* strain N303/Opa$_{50}$culture filtrates.** HUVECs were seeded onto six-well plates in triplicate. The cells were then treated with either filtrates that had been prepared by passing N303/Opa$_{50}$ cultures through a 0.22-μm-pore filter (*panel A*) or 1 μg/ml purified LPS (*panel B*). At the indicated times, total cell lysates were then isolated from HUVECs, and CEACAM1 expression was assessed by Western blot analysis using the CEACAM receptor-specific monoclonal antibody D14HD11. HUVECs cultured in medium alone or in medium supplemented with 10 mg/ml TNF-α were used as negative and positive controls, respectively. The data are representative of at least three independent experiments.

in the levels of expression of these two receptors such as was reported previously (51). Consistent with the previous work, we did see obvious differences in the levels of TLR-2 and TLR-4 message in HDMEC (Fig. 10), suggesting that differences in endothelial cell source and/or growth conditions may effect the relative expression levels of TLR receptors seen. Our data do, however, confirm that TLR-2 and TLR-4 are both expressed by the HUVECs cell lines used in our studies and may thus mediate the NF-κB activation and CEACAM1 expression that results from neisserial infection.

DISCUSSION

In a previous study, we observed that treatment of primary endothelial cells (HUVECs) with the proinflammatory cytokine TNF-α resulted in an induction of CEACAM1 expression and a corresponding increase in adherence and host cellular invasion by Opa-expressing *Neisseria* (13, 15). The localized liberation of TNF-α from sites infected by other pathogens might render the inflamed tissues as targets for colonization by CEACAM-binding strains. Such a phenomenon might help to explain the clinical correlates that imply that an increased risk of invasive meningococcal disease follows viral or other infections of the nasopharyngeal mucosa. TNF-α is present in serum at high levels during disseminated infection (33–35). It may, therefore, also contribute to the rapid progression of invasive neisserial disease as the resulting up-regulation of CEACAM1 expression should facilitate bacterial interactions with the vasculature. Such a detrimental effect of TNF-α expression on the outcome of invasive neisserial infection is supported by the fact that the administration of anti-TNF-α antibodies to infected infant rats protects them against lethal meningococcemia (53). The fact that *Hemeophilus influenzae* is also capable of binding to CEACAM receptors (52) indicates that the up-regulation of CEACAM1 may also influence the outcome of invasive disease by this pathogen. In this context it is interesting to note that the three most important agents of bacterial meningitis, *N. meningitidis, H. influenzae*, and *Streptococcus pneumoniae*

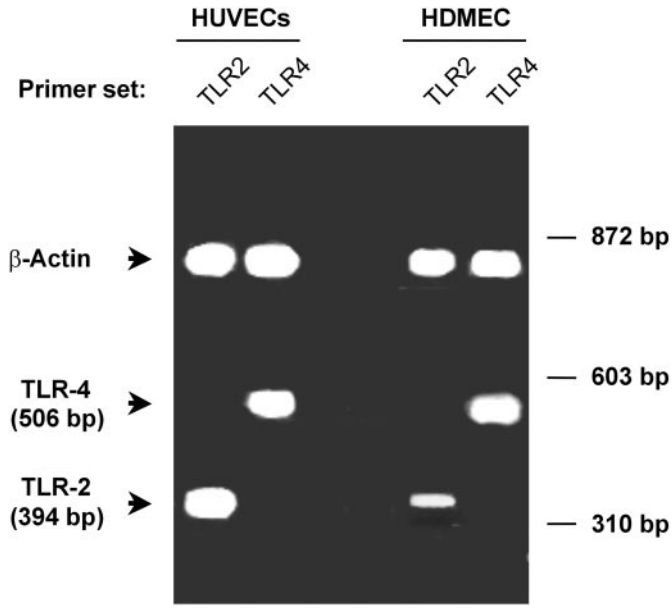

FIG. 10. **Primary endothelial cell expression of TLRs.** TLR-2 and TLR-4 expression was assessed by semiquantitative RT-PCR using total RNA extracted from HUVECs and HDMECs. Primers specific for β-actin were included in the reaction mixture as an internal control. HDMECs were used as an independent source of human TLR mRNA expression to confirm the differences reported previously in the relative expression of TLR-2 and TLR-4 in these cells (40). The identities of PCR products obtained are indicated with *arrows*. The results are representative of at least three independent experiments.

produce IgA1 proteases, and we recently found that neisserial IgA1 protease is a potent stimulator of proinflammatory cytokines in peripheral blood cells (54).

Here we demonstrate that the prolonged exposure of HUVECs to gonococci resulted in an increased level of Opa$_{52}$-dependent bacterial binding, even in the absence of adding

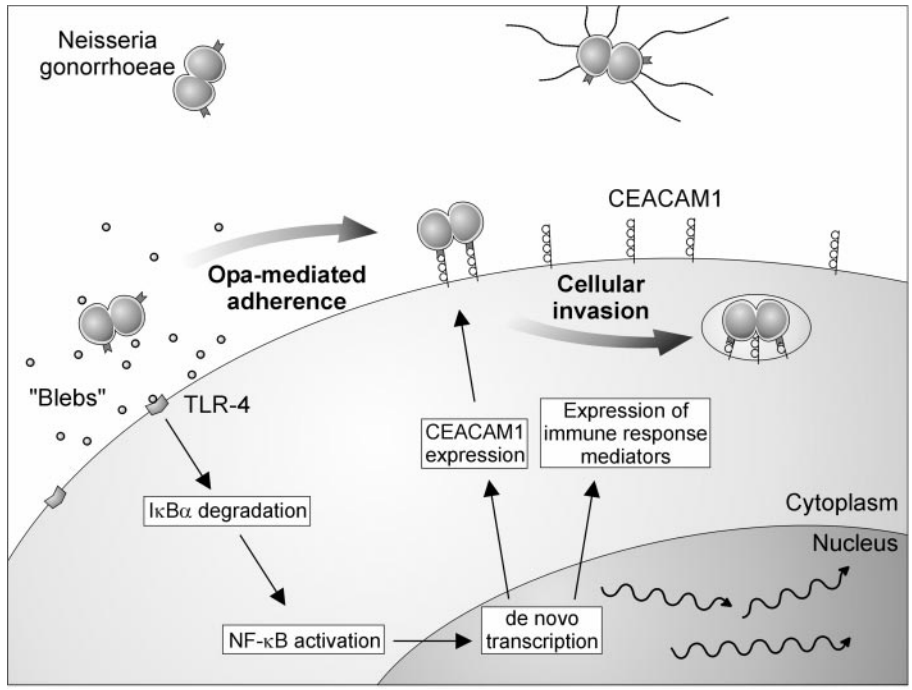

FIG. 11. **Schematic model showing *N. gonorrhoeae* induction of CEACAM1 receptor expression through a NF-κB-dependent pathway.** LPS liberated from adherent or nonadherent infecting gonococci, likely in the form of membrane blebs, triggers a signal cascade through TLR-4 which ultimately leads to the degradation of IκBα. The nuclear translocation of activated NF-κB allows direct induction of the expression of immune mediators, including proinflammatory cytokines (*e.g.* TNF-α, IL-1α) and receptors involved in the recruitment of immune cells, including vascular cellular adhesion molecule 1, E-selectin, and CEACAM1. The newly expressed CEACAM1 allows the gonococci to establish a tight, Opa protein-dependent anchorage to the endothelia and may lead to bacterial uptake into the target cell. A more detailed description of this model is presented under "Discussion."

exogenous cytokine (Fig. 1*A*). This implied that the expression of one or more CEACAM receptor was being up-regulated. Because we have previously observed an induction of TNF-α synthesis in cultured epithelial cells upon neisserial infection (36), we initially speculated that a similar response from endothelial cells could result in an autocrine loop that ultimately resulted in the up-regulation of CEACAM receptor(s). However, we have instead found that anti-TNF-α antibodies have little effect on the induction of CEACAM1 expression (Fig. 4) and that the expression of CEACAM1 transcript is triggered as rapidly as that of any cytokine, including TNF-α (*i.e.* compare Figs. 2*B* and 3). These results imply that CEACAM1 expression is induced directly. Our previous evidence that the immediate early transcription factor NF-κB was central in the inflammatory response of epithelial cells to gonococcal infection (36) thus prompted us to look whether it could also directly control CEACAM1 expression. Gel retardation assays using nuclear extracts isolated from infected HUVECs confirmed that gonococcal infection does activate NF-kB in these cells (Fig. 5), supershift experiments demonstrated that the active NF-κB complex consisted of a heterodimer consisting of the p50 and p65 subunits (Fig. 5), and the inactivation of NF-κB by inhibitors blocked the expression of CEACAM1 (Fig. 6). Together, these data indicate that CEACAM1 is controlled directly by NF-κB. This novel finding implies an important role for this receptor in the endothelial cellular stress response because CEACAM1 would be activated in coordination with proinflammatory cytokines and other receptors that are involved in immune activation. The ability of CEACAM1 to mediate both homotypic and heterotypic interactions with other CEACAM family members might assist in the recruitment of professional phagocytes and lymphocytes that express one or more of these receptors (6, 39). Both CEACAM1 splice variants induced by neisserial infection possess a cytoplasmic immunoreceptor tyrosine inhibitory motif that has been shown previously to arrest the growth of epithelial cells (24, 54). Whether their expression also functions to arrest the growth of infected endothelial cells remains to be determined.

The induction of CEACAM1 by *N. gonorrhoeae* did not require Opa protein expression, suggesting that this effect was not a specific response triggered by bacteria binding to either the host cell HSPG or CEACAM receptors. The ability of a strain to adhere to HUVECs did, however, affect the rate of the host cellular response. This is demonstrated most clearly by comparing the time course of IκB degradation (Fig. 5*B*) and the subsequent activation of NF-κB (Fig. 5*A*) induced by infecting with strain N303, which expresses the Opa_{50} adhesin and can adhere to HUVECs via the HSPG receptors,[2] and N309, which expresses the CEACAM-specific Opa_{52} and thus requires CEACAM1 expression to be induced before effective binding can occur: both responses are induced much more rapidly by N303. It was, therefore, interesting that gonococcal culture filtrates triggered CEACAM1 expression because this indicated that the stimulus was released by the bacteria. *N. gonorrhoeae* actively releases large amounts of membrane blebs, which consist of both protein and lipid components of the outer membrane. Because endotoxin is a potent activator of NF-κB, we tested whether LPS could itself induce CEACAM1 expression. LPS isolated from gonococci (data not shown), *S. typhimurium*, or *E. coli* (Fig. 9) induced CEACAM1 expression with a time course that was similar to that seen with intact bacteria (Fig. 2), indicating that LPS is sufficient for the observed induction of receptor. It is, however, important to consider that although LPS is sufficient to induce CEACAM1 expression, our results do not exclude the possibility that other bacterial components may also contribute to this response. Endothelial cells react to complexes of LPS and serum-soluble CD14 via TLR-4, which induce an IL-1-like signal cascade that ends in the activation of NF-kB (40, 50). Recent studies indicate that the related TLR-2 mediates the cellular responses to microbial lipoproteins (55–59) and to cell wall components other than LPS (60). We thus confirmed that each of these receptors was expressed in our HUVECs by using semiquantitative RT-PCR. Their presence confirms that one or both could trigger a response that ultimately activates NF-κB.

Our results indicate that neisserial infection of primary endothelial cells induces CEACAM1 expression via a pathway that is triggered by the LPS-specific CD14 and TLR-4-dependent activation of NF-κB (Fig. 11). Bacteria that can adhere to

[2] M. Dehio, E. Freissler, K. T. Kreisner, C. Dehio, and T. F. Meyer, manuscript in preparation.

the endothelia by either the HSPG receptor-specific Opa_{50} or, presumably, by pili, trigger this response more quickly than do bacteria that express either no adhesin or CEACAM-specific Opa proteins. These differences likely result from differences in the efficiency of delivery of LPS and/or other soluble effectors because adherent bacteria would presumably cause their local concentration to be higher. The translocation of activated NF-κB into the nucleus directly induces the expression of proinflammatory cytokines such as IL-1, IL-6, and TNF-α, and receptors, which function in the recruitment of immune cells, including the vascular cell adhesion molecule-1, E-selectin (61), and CEACAM1 (this work). Because various cytokines can activate NF-κB, it seems likely that the cytokines expressed by infected endothelial cells will stimulate CEACAM1 expression further. Consistent with this premise, we did observe that anti-TNF-α antibody caused a slight reduction in the level of CEACAM1 produced at later time points (24 h; Fig. 4 and data not shown). This did, however, constitute only a small fraction of the CEACAM1 seen in these cells. The expression of CEACAM1 at the endothelial surface can then mediate an increased Opa/CEACAM1-specific bacterial binding and internalization (Ref. 15 and this work). To our knowledge, this process represents the first example of a bacterial pathogen's ability to autoinduce expression of its host cellular receptor. This remarkable process has important implications for invasive disease because it obviously can mediate interactions between Opa-expressing *Neisseria* and the vasculature *in vivo*. Whether its primary benefit to the bacteria is simple colonization or immune evasion is still unclear; however, the coregulation of various other inflammatory mediators with CEACAM1 presumably means that an immune response is imminent. The induction of CEACAM1 may also be important for other stages of infection because NF-kB is a ubiquitously expressed transcription factor that could presumably allow neisserial induction of CEACAM1 on tissues other than the endothelia. Together, these events clearly represent yet another example of the complex interactions that have evolved between the pathogenic *Neisseria* and humans, their only natural host.

Acknowledgments—We are grateful to Silja Wessler for support in the electrophoretic mobility shift assay and to Andreas Popp for helpful discussions throughout this work.

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J. Biol. Chem. 2001, 276:24331-24340.
doi: 10.1074/jbc.M006883200 originally published online April 16, 2001
