CEACAM is not necessary for Neisseria gonorrhoeae to adhere to and invade female genital epithelial cells

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Summary

Neisseria gonorrhoeae has a repertoire of up to 11 opacity-associated (Opa) proteins that are adhesins. Most Opa proteins adhere to CEACAM antigens and when CEACAM molecules are present on the surface of transfected epithelial cells their binding by Opa is thought to induce invasion of these cells by gonococci. In this study, we investigated whether several malignant epithelial cell lines, normal cervical and fallopian tube epithelial cell cultures, as well as normal fallopian tube tissue express several of the CEACAM molecules, and whether gonococci use these molecules for adherence and invasion of these female genital epithelial cells. A primary cervical cell culture and metastatic cervical cell line ME180 both expressed CEACAM as shown by whole cell ELISA and flow cytometry, and increased the surface expression of total CEACAM during incubation with Opa1 gonococci. Opa1 gonococci both adhered to and invaded these cells; CEACAM-specific monoclonal antibody (MAb) partially abolished this interaction. Two primary fallopian epithelial tube cell cultures, a primary cervical cell culture and two malignant cell lines, HEC-1-B and HeLa, did not express CEACAM nor was CEACAM mRNA present. No evidence of either intracellular or secreted extracellular CEACAM was found with HEC-1-B and HeLa cells. Opa1 gonococci both adhered to and invaded CEACAM non-expressing cells; however, Opa1 gonococcal association with these non-expressing cell lines could not be inhibited with CEACAM-specific MAb. These data show that CEACAM is not always expressed on female genital epithelial cells and is not essential for gonococcal adherence and invasion. However, when CEACAM is expressed, Opa1 gonococci exploit it for the adherence to and invasion of these cells.

Introduction

Several laboratories have reported that Neisseria gonorrhoeae engage receptor molecules of the carcinoembryonic antigen (CEA) family during invasion of human epithelial cells (Chen and Gotschlich, 1996; Bos et al., 1997; Chen et al., 1997; Gray-Owen et al., 1997a, b). The CEA family is encoded by 17 genes; the antigens are divided into two subgroups, CEACAM and pregnancy-specific glycoprotein (PSG; Thompson et al., 1991). The CEACAM subgroup includes four proteins, CEACAM1, CEACAM3, CEACAM6 and CEA, which have been shown to bind to several opacity-associated (Opa) outer membrane proteins of Neisseria gonorrhoeae (Chen and Gotschlich, 1996; Bos et al., 1997; Chen et al., 1997; Gray-Owen et al., 1997a, b). Three of these CEACAM molecules, CEACAM1, CEACAM3 and CEACAM6, are constitutively expressed by neutrophils. CEACAM molecules on the neutrophil surface bind Opa on gonococci and Escherichia coli that have been transformed with gonococcal Opa, and direct the internalization of these bacteria (Gray-Owen et al., 1997b; Chen and Gotschlich, 1996). Gonococci and E. coli that express different Opa proteins differentially bind to and invade HeLa cells that have been transfected with CEACAM1, CEACAM3, CEACAM6 and CEA. T84 cells, a line derived from a lung metastasis of a colon carcinoma, constitutively expresses CEACAM1, CEACAM6 and CEA. T84 cells, a line derived from a lung metastasis of a colon carcinoma, constitutively expresses CEACAM1, CEACAM6 and CEA. T84 cells, a line derived from a lung metastasis of a colon carcinoma, constitutively expresses CEACAM1, CEACAM6 and CEA.

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Results

CEACAM surface expression by epithelial cells

We first used ELISA to determine whether CEACAM molecules were expressed by the malignant cell lines that we and colleagues have used as models for gonococcal invasion, and by three different primary normal cervical cell cultures. As shown in Fig. 1, ME180 was the only malignant female genital epithelial cell line that expressed CEACAM molecules. Monoclonal antibodies (MAbs) CLB-gran/10, which binds CEACAM isoforms 1, 3, 6 and CEA, and ZEA1 (CEACAM6 and CEA and possibly CEACAM1 and 3) bound to ME180 equally well. MAb COL-1, which recognizes only CEACAM3 and CEA, also bound to ME180, but to a lesser extent. MAb 80H3, which is specific for CEACAM8, did not bind to ME180. No binding by any of the four MAbs to HEC-1-B and HeLa cells was detected, and none of the antibodies bound to the primary fallopian tube cells, FT1 or to one of the primary cervical lines, NC6. A second primary cervical cell culture, NC9, bound MAb ZCEA1 and MAb CLB-gran/10, albeit at much lower levels than did ME180 cells. Binding of MAbs COL-1 and 80H3 to NC9 cells was undetected. The colonic adenocarcinoma, HT29, was used as a positive control for CEACAM1, CEACAM3, CEACAM6 and CEA. HT29 bound significantly more MAbs ZCEA1, CLB-gran/10 and COL-1 than did either ME180 or NC9 cells.

We confirmed expression of CEACAM molecules using flow cytometry. ME180 cells bound the same amount of MAb ZCEA1 and MAb CLB-gran/10, and bound significantly less MAb COL-1 (Fig. 2). HEC-1-B and HeLa cells bound none of the MAbs, thus confirming the ELISA data. NC9 primary cervical cells bound MAbs ZCEA1 and CLB-gran/10 equally and confirmed the ELISA data. None of the cells bound MAb 80H3. The colonic adenocarcinoma, HT29, was used as a positive control for CEACAM1, CEACAM3, CEACAM6 and CEA. HT29 bound significantly more MAbs ZCEA1, CLB-gran/10, and COL-1 than did either ME180 or NC9 cells.

CEACAM expression by fallopian tube tissue was investigated by immunohistochemical staining of paraffin sections. Colonic carcinoma tissue was used as a positive control. The failure of MAbs CLB-gran/10 and COL-1 to stain the epithelial cells of any of the four fallopian tube tissues examined showed that none of them expressed CEACAM1, CEACAM3, CEACAM6, or CEA (Fig. 3). MAb CLB-gran/10 bound weakly to an occasional stromal cell in all four of the fallopian tube samples. MAbs CLB-gran/10 and COL-1 bound strongly to colonic carcinoma tissue.
**Gonococcal induced upregulation of CEACAM expression**

As the process of gonococcal invasion occurs over several hours, we looked to see whether CEACAM molecules were being upregulated after exposure of the epithelial cells to gonococci. MS11mkC $P^{-}O^{+}$ were added at a ratio of 25:1 to several of the cell lines and incubated for up to 20 h. The amount of CEACAM expression was determined using ELISA. ME180 cells gradually increased their binding of MAbs COL-1, ZCEA1.

**Fig. 2.** FACS analyses of female genital epithelial cells for CEACAM expression. Cells were labelled with the indicated MAb and detected with FITC-conjugated goat anti-mouse IgG. The fluorescence intensity obtained by these MAbs (solid black area) is compared with isotype-matched controls (solid line). When no CEACAMs were present CEACAM MAbs and the isotype-matched control MAb overlapped. The colonic adenocarcinoma, HT29, was used as a positive CEACAM control.

**Fig. 3.** Immunohistochemistry of four different normal fallopian tube tissues, A-D, and a colonic carcinoma as a positive control. Tissues were incubated with CEACAM binding MAbs as indicated. Positive binding of MAbs was visualized by the brown DAB precipitate. Tissue was then counterstained with hematoxylin (blue staining).
and CLB-gran/10 after exposure to MS11mkC over 20 h (Fig. 4a). After 20 h of incubation with MS11mkC $P^\bullet O^\bullet$ for the length of time indicated, ME180 cells had increased their binding 100% for MAb COL-1, by 50% for MAb ZCEA1 and by 67% for MAb CLB-gran/10. There was no expression of CEACAM8, as determined by the absence of MAb 80H3-binding after 20 h of incubation (results not shown). NC9 primary cervical cells also increased their binding of the same three antibodies (Fig. 4b). MAb COL-1 did not bind to unstimulated NC9, but bound to these cells after 6 h of incubation with MS11mkC and then dropped by 20 h of exposure to MS11mkC. NC9 cells increased their binding of MAb ZCEA1 over the 20 h of incubation with MS11mkC, but the greatest amount of MAb CLB-gran/10 binding to NC9 was seen after 6 h of incubation. Binding of MAb 80H3, COL-1, ZCEA1 and CLB-gran/10 to both HEC-1-B and HeLa cells remained undetectable at 2, 6 and 20 h of exposure to MS11mkC (data not shown).

We used indirect immunofluorescence to investigate the expression of CEACAM by the primary fallopian tube epithelial cells, FT2, in culture. ME180 cells were used as positive controls. FT2 cells did not bind MAb 80H3, COL-1 or ZCEA1 (Fig. 5), consistent with the negative

CEACAM ELISA results for the primary fallopian tube cells, FT1. Binding of MAb COL-1, ZCEA1 and CLB-gran/10 to FT2 remained undetectable after 2, 6 and 20 h of exposure to MS11mkC (results not shown for 2 and 6 h). MAb ZCEA bound the control ME180 cells. Similar staining intensities were seen when ME180 cells were stained with COL-1 and CLB-gran/10 (results not shown). FT2 cells also bound the MAb mixture, AE1/AE3, which binds cytokeratin, an epithelial cell marker, indicating that there is very little contamination of the primary cell culture with non-epithelial cells.

**Intracellular and extracellular expression of CEACAM by epithelial cells**

Although HEC-1-B and HeLa cells, which can be invaded by gonococci, did not surface-express CEACAM, it remains possible that CEACAM could play a role in infection. Because CEA is found in the serum of patients with some malignancies (Chevinsky, 1991), it could be secreted into the extracellular milieu where it could bind gonococci. Alternatively, gonococci could bind intracellular CEACAM molecules after invasion, and this binding could still be essential for the infection process. We therefore analysed lysates and supernatants of ME180, HEC-1-B and HeLa cells for the presence of CEACAM molecules with use of a sandwich ELISA assay. No CEACAM molecules were detected in the growth medium of ME180, HEC-1-B or HeLa cells (results not shown). CEACAM molecules were detected in lysates of ME180 cells, but not in HEC-1-B or HeLa cells. Because ME180 cell lysates contain membrane-expressed proteins, it is unclear if ME180 cells express intracellular CEACAM molecules in addition to the CEACAM found on the cell surface. No evidence of CEACAM expression, whether surface, intracellular or extracellular, was found in HEC-1-B, HeLa, NC6 or normal fallopian tube epithelial cells.

**CEACAM MAb inhibition of gonococcal adherence and invasion**

The role of CEACAM in gonococcal adherence was investigated in the two cell lines that expressed CEACAM, ME180 and NC9. HeLa and HEC-1-B cells were used as CEACAM non-expressing controls. MAb CLB-gran/10, which binds to CEACAM1, CEACAM3, CEACAM6 and CEA, inhibited the adherence of MS11mkC $Opa^-$ organisms to ME180 cells by approximately 30% and to NC9 cells by 40% (Fig. 6A). MAb CLB-gran/10 did not inhibit adherence of MS11mkC organisms to either HEC-1-B or HeLa cells when tested at a MAb concentration 16 times greater than that which inhibited binding of gonococci to ME180 cells.

The role of CEACAM in invasion also was investigated.
MAb CLB-gran/10 was able to inhibit MS11mkC invasion into ME180 cells by about 45%; the antibody was unable to inhibit invasion of either HEC-1-B or HeLa cells by MS11mkC to any degree (Fig. 6B). Although CEACAM may contribute to the invasion process when it is present, it is not necessary for gonococcal invasion of genital epithelial cells.

**CEACAM mRNA expression**

Expression of CEACAM mRNA by the epithelial cell lines was determined by reverse transcription polymerase chain reaction (RT-PCR). RNA was reverse transcribed and then subjected to PCR using primers that would amplify a 460 bp fragment from all members of the CEA family. PCR products were Southern botted and hybridized to an oligonucleotide that binds to CEACAM1, CEACAM3, CEACAM6 and CEA. The two cell lines that expressed CEACAM, ME180 and NC9, were positive for CEACAM PCR product and for hybridization with the CEACAM1,3,5,6 probe (Fig. 7). However, CEACAM non-expressing cell lines, HEC-1-B, HeLa and NC6 were negative for CEACAM PCR product and for hybridization. All cell lines were positive for the control RT-PCR of β-actin, thereby confirming the integrity of the RNA tested. These data confirm the MAb data and show that only those cell lines that express CEACAM molecules express CEACAM mRNA.

Because ME180 and NC9 both increased their expression of CEACAM after exposure of the epithelial cells to gonococci, we used multiplex RT-PCR to semiquantitate mRNA expression of individual CEACAM family members. CEACAM family members were amplified by multiplex RT-PCR along with 18S rRNA as a control. Both ME180 and NC9 constitutively expressed CEACAM1, CEACAM3, CEACAM6 and CEA mRNA and increased the levels of these transcripts in response to MS11mkC. ME180 constitutively expressed many times more CEA and CEACAM3 mRNA transcripts than NC9. ME180 and NC9 both amplified the same two PCR products when CEACAM1 primers were used, indicating they express at least two differentially spliced CEACAM1 transcripts.

Maximum expression of CEACAM1, CEACAM3, CEACAM6 and CEA mRNA transcripts by ME180 was seen after 6 h of exposure of the epithelial cells to MS11mkC and remained elevated after 20 h. Increases in CEACAM1, CEACAM6 and CEA mRNA expression in
NC9 after exposure to MS11mkC $P^-O^+$ were very modest compared with the increases seen in ME180. Increases in expression of CEACAM1, CEACAM6 and CEA mRNA transcripts in NC9 were seen only after 20 h exposure of the epithelial cells to MS11mkC $P^-O^+$. Maximum expression of CEACAM3 by NC9 was seen after 6 h and remained elevated after 20 h.

**MS11mkC invasion of ME180**

Gonococcal invasion of HEC-1-B cells in the presence of serum proceeds only when gonococci express both pili and Opa (Griffiss et al., 1999). As ME180 cells differ from HEC-1-B cells in that they express CEACAMs, we examined how pili and Opa expression affected gonococcal invasion of ME180 cells. Two MS11mkC variants that expressed different Opas, based on different relative electrophoretic mobility using SDS–PAGE, were tested with and without pili, and with and without serum. Optimal invasion of ME180 occurred in the presence of 5% fetal calf serum and when MS11mkC was piliated and expressed Opa (Fig. 9). Piliated Opa negative organisms required serum for optimal invasion of ME180 cells; when no serum was present, MS11mkC $P^-O^+$ organisms invaded ME180 cells only 60% as well as when serum was present (results not shown). When MS11mkC expressed either of the two Opas but not pili, the addition of serum to the invasion assay increased invasion somewhat, but was not significant ($P = 0.333$ and 0.471). MS11mkC $P^-O^+$ invaded ME180 approximately to the same extent as MS11mkC $P^-O^-$, in the presence of serum. When no serum was present, the addition of pili
to MS11mkC expressing either of the two Opas decreased invasion 21% and 19%, respectively, but this decrease was not significant ($P = 0.103$ and 0.131). In the presence of serum, however, the addition of pili to MS11 Opa expressing either of the two Opas decreased invasion 21% and 19%, respectively, but this decrease was not significant ($P = 0.009$ for both Opas).

**Discussion**

Previously it was shown that Opa proteins in the outer membrane of gonococci, or Opa proteins expressed in *E. coli*, bind to CEACAM molecules on the surface of human neutrophils and stimulate phagocytosis of the bacteria by these cells (Chen and Gotschlich, 1996). Similarly, *N. gonorrhoeae* MS11 Opa$_{B-J}$ and *E. coli* transformed to express the same Opa proteins were shown to have increased adherence to and invasion of HeLa cells transfected with CEACAM1, CEACAM3, CEACAM6 or CEA (Chen and Gotschlich, 1996; Bos et al., 1997; Chen et al., 1997; Gray-Owen et al., 1997a, b). Adherence of the Opa-expressing organisms to the CEACAM-transfected HeLa cells could be inhibited by CEACAM antibodies (Chen and Gotschlich, 1996; Chen et al., 1997; Gray-Owen et al., 1997a; Virji et al., 1999). These data imply that most Opa proteins adhere to CEACAM1, CEACAM3, CEACAM6 or CEA on the surfaces of neutrophils and those epithelial cells that constitutively express them, and that such adherence is a necessary part of the invasion process.

We have used HEC-1-B cells to study how gonococci invade cervical cells (Griffiss et al., 1999). Invasion of HEC-1-B cells by MS11$_{MK}$ organisms requires that the bacteria be piliated and express both Opa and paraglobosyl LOS (Griffiss et al., 1999; Wang et al., 1996). In the current study, we investigated whether Opa proteins were engaging CEACAM molecules on the surface of HEC-1-B cells during invasion but found no evidence of CEACAM expression by these cells. We then decided to study whether other epithelial cells of female genital origin surface-express CEACAM molecules. We found that some, but not all, such epithelial cells do express CEACAM. ME180, a cervical carcinoma, and NC9, a primary cervical cell culture, both constitutively express CEACAM1, CEACAM3, CEACAM6 and CEA mRNA and surface-express the corresponding molecules; incubation with MS11mkC P"O" gonococci increased the levels of CEACAM transcripts and the surface expression of total CEACAM in both cell lines. However, HEC-1-B, HeLa, NC6 cells, primary fallopian tube cells in culture and fallopian tube tissue did not express any of the CEACAM molecules. RT-PCR analysis of HEC-1-B, HeLa and NC6 confirmed that no CEACAM mRNA was transcribed in these cells. Furthermore, no evidence of either intracellular or secreted extracellular CEACAM was found with HEC-1-B and HeLa cells.

The finding that many genital epithelial cells do not constitutively express CEACAMs is consistent with the observation of Moncrieff et al. (1984) that only 17 of 24 (71%) normal Papanicolaou cervical cell smears, from women aged 19–59 years (mean = 31 years), stained positive for CEA. The staining of 10 of the 17 positive smears (42% of the total) was weak, whereas seven of the 24 specimens were negative for CEA. Other CEACAM family member molecules were not sought.

Opa$_{B-J}$ have been shown to bind to the N-terminal domains of CEACAM molecules (Bos et al., 1998; 1999; Virji et al., 1999); MAb CLB-gran/10, which binds to the N-terminal domain of CEACAM1, CEACAM3, CEACAM6 and CEA, was able to partially inhibit the adherence of MS11mkC Opa$^+$ organisms to both ME180 and NC9. Presumably, that portion of adherence that was not inhibited by the antibody involved one or more molecular mechanism(s) other than engagement of a CEACAM molecule by Opa. MAb CLB-gran/10 had no effect on adherence of MS11mkC Opa$^+$ gonococci to HEC-1-B and HeLa cells that do not express CEACAM. Additionally, MAb CLB-gran/10 was able to abolish 45% of MS11mkC Opa$^+$ invasion of ME180 but had no affect on invasion of HEC-1B and HeLa cells, thus confirming that gonococcal invasion of HEC-1B, HeLa and those cervical cells that do not express CEACAM does not involve an Opa:CEACAM interaction.

There are several possible ways to explain why increasing the concentration of MAb CLB-gran/10 from 2.5 μg ml$^{-1}$ to 10 μg ml$^{-1}$ increased inhibition of invasion but not of adherence (Fig. 4). We favour the possibility that the bulky monoclonal interfered with engagement of adjacent cell membrane organelles that are involved in the internalization process after the initial adherence step.

In order to show that gonococcal Opa proteins engage CEACAM on the surface of epithelial cells, other
investigators have used transfection to cause HeLa cells to over express individual CEACAM molecules (Bos et al., 1997; Chen et al., 1997; Gray-Owen et al., 1997a, b) or used the colonic carcinoma cell line T84 that constitutively expresses CEACAMs (Wang et al., 1998). We found that HT29, another colonic carcinoma cell line, was also a very high constitutive expresser of CEACAMs compared with ME180, which was the highest expresser among the genital epithelial cells assayed. The normal cervical cell line, NC9, expressed less than half as much CEACAM as ME180. Nonetheless, we were able to show that gonococci do use CEACAM as an adhesin on cervical cells, when it is present, even at low levels of expression.

Cervical and endometrial cells are the primary site of gonococcal infection in the female; cervical infection involves endocervical cuboidal cells rather than ectocervical squamous cells (Draper et al., 1980). It is not known whether women who express CEACAMs on cervical cells also do so on their fallopian tube cells, a secondary site of infection that leads to pelvic inflammatory disease. CEACAM expression by primary fallopian tube cells was not found in this study.

Although gonococci may use CEACAMs to establish infection in women who constitutively express these molecules on cuboidal cervical cells, they would need other mechanisms to infect women who do not. Expression of both pil and Opa (as well as paraglobosyl LOS) are required for optimal invasion of the CEACAM-negative HEC-1-B cells by MS11MK gonococci (Wang et al., 1996; Griffiss et al., 1999). In other studies of CEACAM and gonococcal invasion, pilated bacteria were not used (Chen and Gotschlich, 1996; Bos et al., 1997; Chen et al., 1997; Gray-Owen et al., 1997a, b). Indeed, pil were not needed for invasion of ME180 cells as they were for the HEC-1-B cells, but they did enhance, by a bit less than twofold, the invasion of ME180 cells in the presence of serum. Correspondingly, Opa expression by pilated gonococci, which is required for invasion of HEC-1-B cells, was not necessary for invasion of ME180 (again in the presence of serum). Thus, although CEACAM expression enhances the ability of Opa+ gonococci to adhere to and invade epithelial cells, engagement of CEACAM molecules is not essential for the process of invasion. Gonococci use at least two separate mechanisms, one involving Opa engagement of CEACAMs, the other requiring pil, to ensure their internalization into epithelial cells. The availability of two different mechanisms would seem to ensure that the organism can infect women who are not CEACAM expressers.

Experimental procedures

*Neisseria gonorrhoeae*

Stock cultures of MS11mkC (Schneider et al., 1991; 1995) were maintained in 10% glycerol in Mueller Hinton broth (Difco) at –70°C. The organisms were cultured on gonococcal agar base (Difco) containing 1% IsoVitaleX (Becton Dickinson) at 37°C in candle extinction jars. Eight different Pil Opa+ colonial variants were selected using light microscopy (Norlander et al., 1979) and pooled in equal proportions for the assays described below.

*Epithelial cells*

HEC-1-B, HeLa, ME180 and HT-29 were purchased through ATCC (Rockville). HEC-1-B, an endometrial adenocarcinoma, and HeLa, a cervical epithelioid carcinoma, were both maintained in Eagle’s MEM with Earle’s BSS, non-essential amino acids, sodium pyruvate and 10% fetal calf serum (FCS) at 37°C with 5% CO2. HT-29, a colon adenocarcinoma that is positive for CEACAM expression, and ME-180, an omental metastasis of cervical carcinoma, were maintained in McCoy’s 5a with 10% FCS at 37°C with 5% CO2. Two primary endocervical epithelial cell lines were purchased from Clonetics. These two cell lines were designated NC6 and NC9 for normal cervical followed by the first number of their lot number; they were maintained in KGM-2 (Clonetics) at 37°C with 5% CO2.

Human fallopian tubes were obtained from women who had undergone hysterectomies for various benign conditions at Memorial Medical Center in Springfield, Illinois. Each patient gave consent for tissue to be used in this protocol. The fallopian tubes were harvested and placed in D-MEM/F-12 media supplemented with penicillin/streptomycin, fungizone and defined fetal bovine serum. They were transported to the laboratory as soon as possible after the harvesting and processed immediately. After the adventitial tissues were removed, the tube was cut longitudinally and then cut again into 1–2 mm² pieces (Cooper et al., 1990). The pieces of fallopian tube were placed in a sterile Petri dish containing an enzyme mixture and processed as described previously by Fahey et al. (1998). This method involved the use of an enzyme mixture consisting of 3.4 mg ml⁻¹ pancreatin, 0.1 mg ml⁻¹ hyaluronidase and 1.6 mg ml⁻¹ collagenase. These enzymes were added to a mixture of Hanks’ Balanced Salt Solution with 50 U ml⁻¹ penicillin and streptomycin and 2 mg ml⁻¹ d-glucose added. After incubating the tissue in the enzyme mixture for 2 h at 37°C, the pieces of digested tissue were passed through a series of mesh filters. The epithelial sheets were resuspended in a small amount of D-MEM/F-12 media for cell count and viability. The remainder of the suspension was used for cell culture. A 24-well tissue culture plate was inoculated with 50 µl of the washed cell suspension. One millilitre of the D-MEM/F-12 media was added to the wells. The cell suspension was incubated at 37°C with 5% CO2 for 24 h. After 24 h and thereafter every 24–36 h, fresh media was added with continued incubation at 37°C with 5% CO2. Monolayers took 5–7 days to grow.

*CEACAM antibodies*

MAb ZCEA1 recognizes CEA and CEACAM6 and possibly has cross-reactivity with CEACAM1 and CEACAM3 (Zymed). MAb CLB-gran/10 recognizes CEACAM1, CEACAM3, CEACAM5 and CEACAM6 and possibly has cross-reactivity with CEACAM1 (Zymed).
CEACAM6 and CEA (Grunert et al., 1995; Research Diagnostics). MAb 80H3 recognizes CEACAM8 (Biodesign). COL-1 recognizes CEACAM3 and CEA (NeoMarkers) Rabbit polyclonal anti-CEA was purchased from DAKO.

**Elisa**

All epithelial cell lines were washed two times in phosphate-buffered saline (PBS) before loading approximately 1.5 x 10^6 cells per well of Immulon 2 (Dynatech) 96-well dishes. The wells were allowed to air-dry overnight in a Laminar flow hood. The wells were blocked with 2% BSA in PBS 0.05% Tween 20 for 2 h at 37°C. Wells were washed two times with PBS and then incubated for 2 h at 37°C with 400 ng ml^{-1} of primary MAb. The wells were washed 4–5 times with PBS that contained 0.05% Tween 20 and then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma) for 2 h at 37°C. The wells were washed 4–5 times with PBS 0.05% Tween 20 and then developed with TMB substrate system (Sigma) at 37°C for approximately 1 h. The development was stopped with the addition of 0.5 M H_2SO_4 followed by reading at A_450 nm.

ELISAs in which the epithelial cells were first exposed to gonococci were set up as follows. Epithelial cells (5 x 10^5) were loaded into 96-well cell culture dishes and incubated at 37°C with 5% CO_2 for 40 h. Cells were washed once with PBS. Next, 1.25 x 10^6 MS11mkC P’O’ in 100 µl of fresh media was added and the cells were incubated at 37°C with 5% CO_2 for 2–20 h. The cells were washed four times with PBS to remove non-adherent bacteria and then air-dried for 2 h in a Laminar flow hood.

For sandwich ELISAs, 96-well dishes were coated with 10 µg ml^{-1} polyclonal anti-CEA and incubated at 4°C overnight. Wells were washed three times with PBS that contained 0.05% Tween 20 followed by blocking with BSA as above. Cell lysates were prepared by suspending washed epithelial cells at 5 x 10^6 ml^{-1} in 1 mM EDTA, 1 mM phenylmethanesulphonyl fluoride (PMSF) and 0.05% Tween 20. The cells were heated to 72°C for 5 min The cell lysates were centrifuged for 30 s and the supernatants were incubated at 37°C for 1 h with anti-CEA coated 96-well dishes. The presence of CEACAM was detected with primary monoclonal antibodies as described above.

**Fluorescence assays**

For flow cytometric analyses, 10^6 cells were incubated for 1 h with 100 µl of 10 µg ml^{-1} primary antibody diluted in PBS that contained 0.2% BSA. The cells were washed three times in PBS/BSA and then incubated for 1 h in 100 µl of a 1:250 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Zymed). The cells were washed three times in PBS/BSA and then fixed for 30 min in 1% paraformaldehyde. For controls, isotype-matched control MAbs, IgG1 and IgG2a (Caltag), were incubated with the cells. Immunofluorescence was detected using a Becton Dickinson FACScan flow cytometer equipped with LYSIS II software for data acquisition and analysis.

For indirect immunofluorescence analyses, the cells were grown to 50% confluency on tissue culture chamber slides and fixed at -20°C in methanol for 10 min. After two washes with PBS, the cells were blocked with 0.2% BSA in PBS for 15 min and then incubated with 1 µg ml^{-1} MAb for 1 h at room temperature. The cells were washed twice with PBS and reacted with a 1:200 dilution of Alexa 488 conjugated goat anti-mouse IgG (Molecular Probes) for 1 h at room temperature. After three washes with PBS, the cells were visualized using fluorescence microscopy.

**Immunohistochemistry**

Sections of formalin-fixed and paraffin-embedded fallopian tissue and colonic carcinoma (BioGenex) were deparaffinized in xylene and rehydrated in 100% ethanol through 75% ethanol. Sections were rinsed in milli-q H_2O, incubated in 3% H_2O_2 in methanol for 30 min and then rinsed in PBS. Sections were heated in a microwave oven in 10 mM citrate buffer pH 6.0 on high-power setting until the solution reached a rapid boil and then for 10 min on medium-low-power setting according to the method of Shi et al. (1991). The sections in solution were cooled at room temperature for 30 min. Sections were rinsed in PBS and non-specific antibody was blocked by incubation of the sections in 10% horse serum for 30 min at room temperature. Blocking serum was removed by blotting, and the sections were incubated with 2 µg ml^{-1} MAb COl-1 or CLB-gran/10 for 1 h, rinsed in PBS, incubated with biotinylated secondary horse anti-mouse antibody for 30 min and rinsed in PBS. Sections were then incubated for 30 min with Vectastain Elite peroxidase-avidin complex (ABC reagent, Vector Laboratories), rinsed in PBS and incubated for 5 min in DAB substrate (Vector Laboratories). Sections were rinsed in dH_2O before being counterstained with hematoxylin (BioGenex) and examined using light microscopy.

**Adherence and invasion assays**

For adherence assays, 5 x 10^4 cells were plated in 96-well dishes and incubated at 37°C in 5% CO_2 for 24–40 h until confluent. Cells were washed twice with appropriate medium. Fresh medium (100 µl) that contained 0–40 µg ml^{-1} CLB-gran/10 or isotype-matched control MAb was added to the cells and allowed to incubate at 37°C for 10 min in 5% CO_2. Next, 1.25 x 10^6 MS11mkC P’O’ was added to the cells and incubation continued for 2 h at 37°C with 5% CO_2. The cell monolayers were washed four times with PBS. Adherent bacteria were quantified by suspending the cells in 1% saponin and then plating appropriate dilutions on GC medium. After 24 h incubation at 37°C in a candle extinction jar, CFU were counted. The significance of differences was assessed using the Student’s t-test for independent population means.

Invasion assays were carried out in much the same way, except that the bacteria were incubated with the cell monolayers for 5 h, washed three times with PBS and then incubated with 50 µg ml^{-1} gentamicin in the appropriate medium for 1.5 h at 37°C in 5% CO_2. The monolayers were washed three times with PBS followed by the addition of saponin solution and plating as above.
**Rt-pcr**

Total CEACAM mRNA was isolated and analysed as follows. Total cellular RNA was isolated using the acid guanidinium thiocyanate method (Totally RNA reagents) according to the manufacturer’s instructions (Ambion). Total RNA was treated with RNase-free DNase (Promega) as per the manufacturer’s instructions. RNase-free DNase was inactivated by the addition of EGTA to a final concentration of 1 mM and heating to 65°C before reverse transcription. Ten microliters of the DNase digested RNA reaction was used in 20 μl RT reactions. Final reaction conditions were 0.5 μg total RNA, 1 × PCR buffer (Perkin-Elmer), 5 mM MgSO4, 0.5 mM dNTPs, 0.5 μg oligo-dT (Promega), and 200 U of Superscript II (Gibco BRL) reverse transcriptase. The mixtures were incubated at 42°C for 50 min and then heated to 75°C for 10 min.

Each PCR reaction contained 10 μl of the RT reaction, 1 × PCR buffer, 1 mM MgSO4, 100 mM dNTPs, 30 pmol of each primer and 2.5 U of AmpliTaq Gold (Perkin Elmer) in a 50 μl reaction. The CEACAM primers, forward 5’ GGCA-GAGGCTCTGCTGCTCAC (Thompson et al., 1993) and reverse 5’ CAGGTGAAGGCCACAGCATC, will amplify a 460 bp fragment from all CEACAM family members. The reaction mixtures were heated to 95°C for 6 min and then cycled 35 times at 95°C for 1 min, 57°C for 1 min, and 72.5°C for 1.5 min and given a final elongation at 72.5°C for 10 min. Aliquots of the PCR reactions were electrophoresed through agarose gel and visualized using ethidium bromide. The DNA in the gel was transferred to nylon membrane and hybridized with a CEACAM1,3,5,6 specific oligo as per published protocols (Cuby et al., 1992). The specific oligo, 5’ AGGAACCTCAACAGCTACCCCA was labelled with 32P and hybridized with the DNA for 16 h at 45°C.

Individual CEACAM family member multiplex RT-PCR was performed as follows. First, 5 μg of total RNA was reverse transcribed in a 20-μl reaction according to the manufacturer’s instructions. Next, 1 μl (0.25 μg starting RNA) was amplified with use of the primers listed in Table 1. 18S RNA was co-amplified using 18S RNA alternative primers:competimers in a 1:9 ratio (Ambion). 18S RNA primer:competimers were added to the PCR reactions during the last 16 cycles. CEACAM1 and CEA were co-amplified with a total of 26 cycles and CEACAM3 to the PCR reactions during the last 16 cycles. CEACAM1 and 18S rRNA were co-amplified with a 1:9 ratio (Ambion). 18S rRNA primer:competimers were added to the PCR reactions during the last 16 cycles. CEACAM1 and 18S rRNA were co-amplified with a total of 28 cycles, CEACAM6 and CEA were co-amplified with a total of 26 cycles and CEACAM3 was amplified with a total of 40 cycles.

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**Table 1. Primer sets for amplification of specific CEACAM family members by RT-PCR.**

| Transcript | Primer | Sequence | Fragment length(s) (bp) | Reference |
|------------|--------|----------|-------------------------|-----------|
| CEACAM1    | A-for  | 5’ ACAGTCAAGCACGATCATAG | 242, 477 | Wang et al. (1998) |
|            | A-rev  | 5’ ATCTTGTAGGTGGGTCATT  |            |           |
| CEACAM3    | CEA-237| 5’ AGGAACCTCAACAGCTACCCCA | 394      | This study |
|            | D-rev  | 5’ AGTGAGAGGGGACACATC  |            |           |
| CEACAM6    | C-for  | 5’ TTCTTCTACTGCCCACAC  | 474       | Thompson et al. (1993) |
|            | C-rev  | 5’ GTTCTTTTGTGGCTGGAAGTA |            |           |
| CEA        | E-for  | 5’ CCATGAGTTCTCCTCG  | 641       | Thompson et al. (1993) |
|            | E-rev  | 5’ GTAGCTTGCTGTCATT  |            |           |

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