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CD66 carcinoembryonic antigens mediate interactions between Opa-expressing *Neisseria gonorrhoeae* and human polymorphonuclear phagocytes

Scott D.Gray-Owen[1], Christoph Dehio[1], Anja Haude[1], Fritz Grunert[2] and Thomas F.Meyer[1,3,4]

[1]Max-Planck-Institut für Biologie, Abteilung Infektionsbiologie, Spemannstrasse 34, 72076 Tübingen, [2]Universität Freiburg, Immunbiologisches Institut, Stefan-Meier-Strasse 8, 79104 Freiburg and [3]Max-Planck-Institut für Infektionsbiologie, Abteilung Molekulare Biologie, Monbijoustrasse 2, 10117 Berlin, Germany

[4]Corresponding author
e-mail: sinfbio@mpib-tuebingen.mpg.de

Colonization of urogenital tissues by the human pathogen *Neisseria gonorrhoeae* is characteristically associated with purulent exudates of polymorphonuclear phagocytes (PMNs) containing apparently viable bacteria. Distinct variant forms of the phase-variable opacity-associated (Opa) outer membrane proteins mediate the non-opsonized binding and internalization of *N.gonorrhoeae* by human PMNs. Using overlay assays and an affinity isolation technique, we demonstrate the direct interaction between Opa_{52}-expressing gonococci and members of the human carcinoembryonic antigen (CEA) family which express the CD66 epitope. Gonococci and recombinant *Escherichia coli* strains synthesizing Opa_{52} showed specific binding and internalization by transfected HeLa cell lines expressing the CD66 family members BGP (CD66a), NCA (CD66c), CGM1 (CD66d) and CEA (CD66e), but not that expressing CGM6 (CD66b). Bacterial strains expressing either no opacity protein or the epithelial cell invasion-associated Opa_{50} do not bind these CEA family members. Consistent with their different receptor specificities, Opa_{52}-mediated interactions could be inhibited by polyclonal anti-CEA sera, while Opa_{50} binding was instead inhibited by heparin. Using confocal laser scanning microscopy, we observed a marked recruitment of CD66 antigen by Opa_{52}-expressing gonococci on both the transfected cell lines and infected PMNs. These data indicate that members of the CEA family constitute the cellular receptors for the interaction with, and internalization of, *N.gonorrhoeae*.

Keywords: adhesin/CEA family/gonococci/neutrophil/receptor

Introduction

Neisseria gonorrhoeae is a strict parasite of humans whose presence can be manifested by signs ranging from asymptomatic disease to the massive inflammatory response leading to a purulent urethral or cervical discharge typical of gonorrhea. Upon entering the urogenital tract or other host tissues, the gonococcal pilus is responsible for early interactions with human epithelial cells in a tissue- and species-specific manner (McGee *et al.*, 1983; Rudel *et al.*, 1992). Once attached, secondary binding interactions which are mediated by members of the colony opacity-associated Opa protein family are formed. These interactions allow the organism to generate a tight association with host tissues that can characteristically result in the invasion of epithelial cells in culture (Makino *et al.*, 1991; Weel *et al.*, 1991). Only one of the 11 distinct variant Opa proteins of a given strain is capable of mediating the adherence to and invasion of epithelia cells (Makino *et al.*, 1991; Kupsch *et al.*, 1993; van Putten and Paul, 1995), and cell surface-associated heparan sulfate proteoglycans are involved in this binding (Chen *et al.*, 1995; van Putten and Paul, 1995). Rearrangement of the actin cytoskeleton is necessary to facilitate bacterial entry via a process which resembles phagocytosis more closely than macropinocytosis (Grassmé *et al.*, 1996). The variation of expressed Opa proteins is mediated by RecA-independent rearrangements in the coding repeat (CR) sequence which lead to translational reading frame shifts by modifying the number of repeat units between the translational start codon and the mature N-terminus of Opa (Stern *et al.*, 1986). The phase variation of individual *opa* loci has been estimated to occur at a frequency of 10^{-3} *in vitro*, thus generating a heterogeneous population of gonococcal cells with members producing none, one or multiple distinct Opa protein(s) at any time. Based upon its estimated mol. wt of 30 kDa, the epithelial cell-binding protein was historically referred to as Opa_{30} and is encoded by the *opaC* chromosomal locus (Bhat *et al.*, 1991; Bihlmaier *et al.*, 1991). The chromosomal *opa* genes are, however, subject to sequence variation due to recombination between separate loci (Stern *et al.*, 1986; Connell *et al.*, 1988; Bhat *et al.*, 1991), thus leading to different nomenclature being used to denote common *opa* loci by different research groups (Bihlmaier *et al.*, 1991; Belland *et al.*, 1992; Kupsch *et al.*, 1993). Since recombinant strains expressing characterized loci were employed in this study, the terminology of Kupsch *et al.* (1993) will be employed throughout the remainder of this manuscript, and the epithelial cell invasion-associated Opa_{30} protein will thus be referred to as Opa_{50}.

Primary urogenital colonization by the gonococci can trigger a urogenital response which leads to the production of a purulent exudate consisting primarily of polymorphonuclear granulocytes (PMNs) typical of symptomatic gonorrhea. Examination of clinical specimens reveals that many phagocytes in this discharge are associated with gonococci (Rest and Shafer, 1989; Apicella *et al.*, 1996). Despite this fact, little is known about the specific interactions which occur between PMNs and this pathogen. At the time of previous studies on the role of pili and Opa proteins in this process (Ofek *et al.*, 1974; Dilworth *et al.*, 1975; Gibbs and Roberts, 1975), the full repertoire of

these variable factors was not yet described. However, most Opa^+ variants of *N.gonorrhoeae* adhere to neutrophils and induce a chemiluminescence (CL) response whereas Opa^- bacteria do not (King and Swanson, 1978; Virji and Heckels, 1986; Fischer and Rest, 1988). The non-opsonized phagocytosis of *N.gonorrhoeae* and the subsequent granulocytic respiratory burst can be inhibited by Opa-specific monoclonal antibodies (Elkins and Rest, 1990), suggesting that Opa function was necessary for these processes to occur. Using recombinant gonococcal and *Escherichia coli* strains constitutively expressing individual Opa protein variants, independent groups have shown that Opa_{50} mediates significantly less binding to human PMNs in the absence of divalent cations than the other Opa proteins (Belland *et al.*, 1992; Kupsch *et al.*, 1993), while in the presence of divalent cations significant binding of Opa_{50} is observed (D.R.Lorenzen, J.Pandit, D.Gunther, E.Brandt, T.Rudel, and T.F.Meyer, in preparation).

Farrel and Rest (1990) have reported that the levels of gonococcal adherence to human neutrophils is increased significantly upon degranulation, leading to the extracellular release of secondary and tertiary granular contents. The carcinoembryonic antigen (CEA) subgroup of the CEA family consists of seven distinct members, four of which are expressed on PMNs (Thompson *et al.*, 1991). CEA protein (CD66e), the defining member of the CEA family, is a widely used tumour marker of 180–200 kDa which is expressed on colonic mucosa (Thompson *et al.*, 1991) but is absent from granulocytic cells. The CEA members which are expressed on granulocytes are found at significant levels and migrate to apparent mol. wts of ~180 (CD66a: biliary glycoprotein, BGP) and 90 kDa (CD66b: CEA gene family member 6, CGM6, and CD66c: non-specific cross-reacting antigen, NCA) based upon SDS–PAGE analysis. Although Western blot analysis has not led to detection of the 30–35 kDa CGM1 protein (CD66d: CEA gene family member 1) on freshly isolated human cells, its expression on granulocytes is inferred based upon the presence of mRNA transcripts and can be detected using FACScan analyses with a CGM1-specific monoclonal antibody (Nagel *et al.*, 1993). In the present study, we have used recombinant gonococci and *E.coli* expressing defined Opa proteins to identify individual members of the CEA family of proteins which are capable of interacting specifically with those Opa proteins which mediate binding to human PMNs. Transfected HeLa cell lines expressing individual members of the CEA family were used to analyse Opa-mediated interactions with each member in isolation. These studies have revealed that gonococcal Opa_{52}-mediated binding of the CEA family members correlates with their ability to invade the corresponding HeLa cell line. Based upon sequence homologies which exist between members of this protein family, the data presented here allow the region recognized by Opa to be circumscribed to the N-terminal 108 amino acids of these antigens.

Results

Gonococcal overlay of electroblotted PMN proteins reveals Opa_{52}-mediated binding to specific proteins

The expression of various Opa proteins by *N.gonorrhoeae* has been shown previously to significantly affect the level and outcome of binding interactions which occur between this bacteria and human polymorphonuclear phagocytes (Belland *et al.*, 1992; Kupsch *et al.*, 1993). Non-piliated, Opa^- gonococci do not interact with PMNs in the absence of opsonin, while the expression of either Opa_{50} or Opa_{52} does allow significant binding to occur (Kupsch *et al.*, 1993). Opa_{50} has been shown previously to bind human epithelial cells via cell surface-associated proteoglycan receptors (Chen *et al.*, 1995; van Putten and Paul, 1995), and it does mediate less binding to PMNs than do the other 10 Opa proteins (Belland *et al.*, 1992; Kupsch *et al.*, 1993). Based upon *in vitro* studies, no significant difference is seen between the abilities of the other 10 Opa proteins expressed by *N.gonorrhoeae* MS11 to mediate binding and internalization by PMNs (Kupsch *et al.*, 1993). Using an *in vivo* model of experimental gonorrhea, Swanson and co-workers (1988) did, however, find that gonococci recovered from male volunteers who had received an intraurethral inoculation of a piliated, Opa^- strain were predominantly opaque. Interestingly, immunoblot analysis of these bacteria revealed that >60% expressed the Opa_{52} protein homologue. Based upon these results, our present analysis of Opa-mediated interactions with human PMNs focused upon this protein. Opa_{60}-expressing bacteria were employed in parallel studies in order to determine if similar interactions are mediated by more than one Opa protein. Although not presented with this study, results obtained when using Opa_{60} bacteria were consistently indistinguishable from those expressing Opa_{52}.

Previous studies have indicated that gonococcal binding to PMNs is strongly enhanced by the release of subcellular secondary or tertiary granule components to the extracellular milieu (Densen and Mandell, 1978; Farrel and Rest, 1990). In an attempt to test the hypothesis that these interactions are mediated by a proteinaceous receptor expressed on the surface of neutrophils, whole cell lysates from freshly isolated human PMNs were prepared. Following boiling in the presence of reducing agent, component proteins separated by SDS–PAGE and electroblotted onto Immobilon P transfer membranes were probed using intact, biotin-labelled recombinant gonococci expressing defined Opa proteins. Transparent colonies of the parental non-piliated *N.gonorrhoeae* MS11 strain N302, which contains a deletion in the *opaC* locus encoding the phase-variable chromosomal homologue of the recombinant Opa_{50} (Kupsch *et al.*, 1996), were used as an Opa^- control. Complementation of this strain with the stably expressed recombinant genes encoding Opa_{50} or Opa_{52} was described previously (Kupsch *et al.*, 1993). Opa protein expression patterns were monitored routinely by immunoblot analysis using the Opa-specific monoclonal antibody 4b12/CII in order to confirm that interactions detected could be attributed to the constitutively expressed recombinant Opa proteins (data not shown). Adherent bacteria were detected by subsequent incubation of the blots with a streptavidin–horseradish peroxidase conjugate (SA-HRP), and indicated that distinct bands representing proteins of ~90 and 180 kDa were bound by *N.gonorrhoeae* expressing Opa_{52}, whereas no binding of transparent or Opa_{50}-expressing gonococcal strains was detected using this technique (Figure 1A).

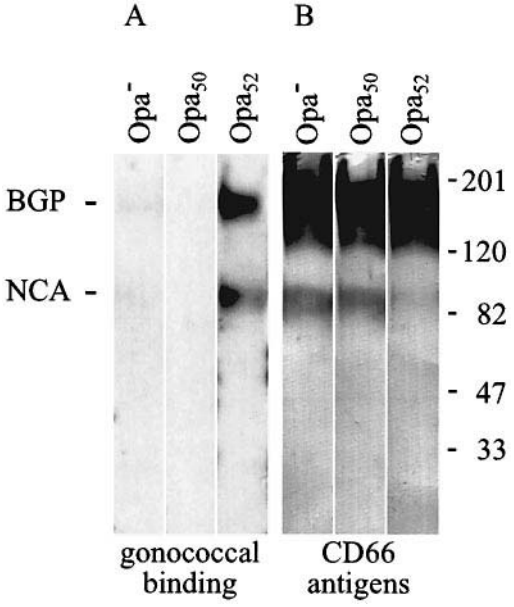

Fig. 1. Gonococcal binding to electrophoretically separated PMN components by bacterial overlay assay. Whole cell extracts were prepared from freshly isolated human polymorphonuclear cells and component proteins from a proportion corresponding to 1×10^5 cells/lane were separated on a 4–15% gradient SDS–PAGE gel. After electroblotting, the Immobilon-P transfer membrane was blocked using PBS/Mg/Ca buffer containing 5% skimmed milk powder and then incubated with biotin-labelled transparent (Opa$^-$), Opa$_{50}$- or Opa$_{52}$-expressing *N.gonorrhoeae* diluted in blocking buffer. (**A**) Detection of bound, biotinylated bacteria using streptavidin–horseradish peroxidase conjugate; (**B**) subsequent probe of overlaid blots using the CD66-specific monoclonal antibody CLB/gran10.

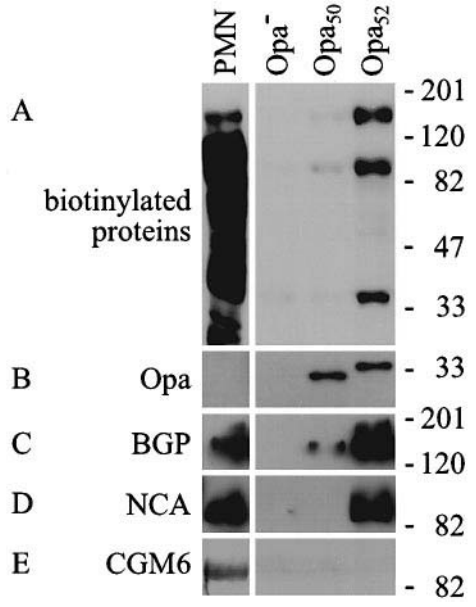

Fig. 2. Specific isolation of surface-biotinylated PMN proteins by defined Opa-expressing gonococcal strains. Triton X-100-soluble extracts were prepared from surface-biotinylated polymorphonuclear cells and then incubated with intact transparent (Opa$^-$), Opa$_{50}$- or Opa$_{52}$-expressing *N.gonorrhoeae.* After washing, the bacteria were recovered by centrifugation, resuspended into PBS and then 2× SDS–PAGE sample buffer containing 2-mercaptoethanol and boiled for 10 min. Replicate blots were then probed using: (**A**) streptavidin–horseradish peroxidase; (**B**) Opa-specific mAb 4b12/CII; (**C**) CD66 family-specific mAb gran10; (**D**) NCA-specific mAb 9A6; and (**E**) CGM6-specific mAb 80H3.

Opa$_{52}$ mediates the specific binding of distinct members of the CEA family of receptor proteins

Previously, receptors for *Yersinia pseudotuberculosis* invasin and *Listeria monocytogenes* internalin have been identified on epithelial cells using a purification protocol based upon the preparation of affinity matrices prepared using the purified bacterial proteins (Isberg and Leong, 1990; Mengaud *et al.*, 1996). Such an approach was facilitated by the previous characterization of soluble forms of the affinity ligands which maintained a functional conformation throughout purification and receptor isolation procedures. Neisserial Opa proteins are a family of integral membrane proteins which are postulated to pass through the outer membrane of *N.gonorrhoeae* eight times in order to produce four surface-exposed regions thought to mediate Opa function (Bhat *et al.*, 1991). This topology suggests that membrane insertion is a prerequisite for the maintenance of secondary structure(s) necessary for Opa-mediated interactions to occur. In order to identify the cellular receptor for Opa$_{52}$ on human PMNs, an affinity purification scheme employing intact gonococci expressing defined Opa variants was proposed so that the native Opa conformation could be maintained. For this procedure, freshly isolated PMNs were surface labelled using the membrane-impermeant biotinylation reagent NHS-LC-biotin. In an attempt to also label proteins which may be released to the cell surface from intracellular granules, PMN degranulation was induced by the addition of phorbol myristate acetate (PMA) during the biotinylation reaction. After labelling, detergent-soluble proteins were extracted with Triton X-100 and then incubated with intact, recombinant gonococci expressing defined Opa proteins. After extensive washing by successive rounds of centrifugation and resuspension of the bacterial pellet, recovered proteins were separated by electrophoresis and probed using Western blot analysis.

The detection of biotinylated proteins by using SA-HRP revealed that proteins having apparent mol. wts of ~35, 90 and 180 kDa were isolated by Opa$_{52}$-expressing *N.gonorrhoeae* (Figure 2A). The recovered yield of each of these three proteins is typically at least 20–50 times greater than that obtained when using either Opa$^-$- or Opa$_{50}$-expressing gonococcal strains. This level of enrichment is consistent with the well described existence of variant Opa proteins being expressed by ~1% of the bacterial population in *in vitro* culture (Swanson *et al.*, 1988; Bhat *et al.*, 1991; Makino *et al.*, 1991; Kupsch *et al.*, 1993). In order to ascertain whether differences in recovered protein could be attributed to differences in recovered bacteria, and to confirm that Opa expression was consistent with that expected from these strains, replicate blots were also probed for Opa (Figure 2B) or stained using Coomassie brilliant blue (data not shown). As illustrated by the level of Opa$_{50}$ and Opa$_{52}$ present in the final bacterial pellet, no significant difference in bacterial protein recovery is evident, thus implying that the enhanced recovery of biotin-labelled protein from PMNs is attributable to their expression of distinct Opa proteins.

The pattern of proteins recovered by Opa$_{52}$-expressing gonococci suggested that they may be members of the CEA family of receptor proteins which are expressed on

granulocytic cells (Thompson *et al.*, 1991). Western blot analysis of the recovered samples using the CD66 epitope-specific CLB/gran10 monoclonal antibody revealed immunoreactive bands which co-migrate with the 180 (Figure 2C) and 90 kDa (data not shown) biotinylated proteins. Consistent with this, an obvious reduction in the 90 kDa protein detected by immunoblotting of total PMN lysates with CLB/gran10 antibodies was evident when the blot was pre-incubated with Opa_{52}, but not Opa_{50} or transparent bacteria in an overlay assay (Figure 1B).

The absence of CEA expression on PMNs indicates that the recovered high molecular weight CLB/gran10-reactive protein is BGP (Figure 2C). Although NCA and CGM6 are indistinguishable based solely upon their electrophoretic mobilities, CGM6 is not immunoreactive with CLB/gran10, thus indicating that Opa_{52} also binds NCA. In order to ascertain whether the 90 kDa band seen in Figure 2A actually consisted of two co-migrating CEA family members, replicate blots were probed with monoclonal antibodies 9A6 and 80H3 which specifically recognize NCA and CGM6, respectively. This analysis indicated that NCA (Figure 2D) but not CGM6 (Figure 2E) are isolated by Opa_{52}-expressing gonococci. Significantly, the levels of BGP (Figure 2C) and NCA (Figure 2D) found associated with the Opa_{52}-expressing gonococci are indistinguishable from that seen in the applied PMN extract, suggesting that nearly complete recovery of these antigens was achieved. In contrast, the presence of significant amounts of CGM6 in the PMN extract and its complete absence from the bacterial pellet demonstrates the specificity of the affinity protocol employed and also confirms that the presence of BGP and NCA was not due to the non-specific isolation of incompletely solubilized membrane sheets remaining in the labelled granulocytic extract. Although co-migrating with the expected molecular weight of CGM1, the 35 kDa biotinylated band could not be confirmed to be identical to CGM1 by cross-reactivity with antibodies. This might be due to the fact that mAbs CLB/gran10 and Col-1, which have been used previously to measure the expression of CGM1 on intact neutrophils, do not react with CGM1 after Western blotting (Nagel *et al.*, 1993). Whether the 35 kDa biotinylated protein from PMNs is CGM1 or is instead an as yet unidentified protein which is co-isolated by Opa_{52} but which lacks the CD66 epitope is thus uncertain based upon this analysis. Significantly, however, incubation of Opa_{52}-expressing gonococci with Triton X-100-soluble extracts from transfected HeLa cells expressing CGM1 also results in the isolation of a protein of ~35 kDa which is not isolated from HeLa cell lines not expressing CGM1 (data not shown), thus indicating that CGM1 is recognized and can be bound by Opa_{52}.

Opa_{52} mediates neisserial attachment and entry into transfected HeLa cell lines expressing recombinant CEA protein family members

Further evidence for the specificity of gonococcal Opa_{52}-mediated binding *in vivo* was achieved using stably transfected HeLa cell lines which express individual members of the CD66 family. Although the CEA is not expressed on PMNs, it does possess significant homologies to the other CD66 family members, and a CEA-expressing cell line was therefore included in these studies. Recombinant gonococci expressing Opa_{50}, Opa_{52} or no Opa protein were incubated with individual cell lines and adherence levels were analysed by phase contrast microscopy (Figure 3) and by plating of bacteria recovered after saponin lysis (data not shown). The levels of associated bacteria seen in Figure 3 are indicative of results obtained using the quantitation of colony-forming units recovered. As is illustrated, transparent (Opa^-) gonococci show negligible adherence to all six HeLa cell lines, while Opa_{50}, which mediates epithelial cell binding via cell surface-associated proteoglycan receptors (Chen *et al.*, 1995; van Putten and Paul, 1995), results in significant binding irrespective of CD66 expression. CD66 expression does, however, clearly affect attachment of the Opa_{52}-expressing gonococci. Consistent with the receptor isolation studies (Figure 2), Opa_{52} does not mediate binding to HeLa cells expressing CGM6 or to HeLa-Neo, a control cell line transfected with the empty expression vector, yet significant binding is evident when either CGM1, NCA, BGP or CEA are expressed (Figure 3). Whether differences in the level of bacterial

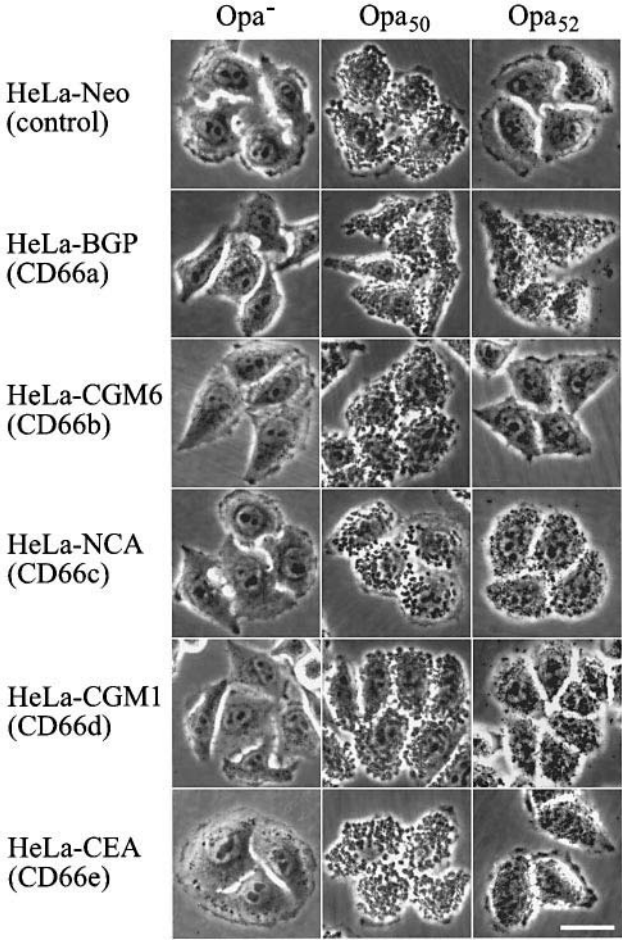

Fig. 3. Opa-mediated binding of *N.gonorrhoeae* strains to stably transfected HeLa cell lines expressing various members of the CD66 family. HeLa cell lines stably transfected with an empty vector control (Neo), or expressing either BGP (CD66a), CGM6 (CD66b), NCA (CD66c), CGM1 (CD66d) or CEA (CD66e) were infected with *N.gonorrhoeae* strains expressing either no Opa protein (Opa^-), the epithelial cell invasion-associated Opa protein (Opa_{50}) or an Opa protein associated with binding to PMNs (Opa_{52}) for 3 h at 37°C and 5% CO_2 before washing and fixing. Phase-contrast pictures are presented. A scale bar corresponding to 20 μm is indicated in the lower right picture.

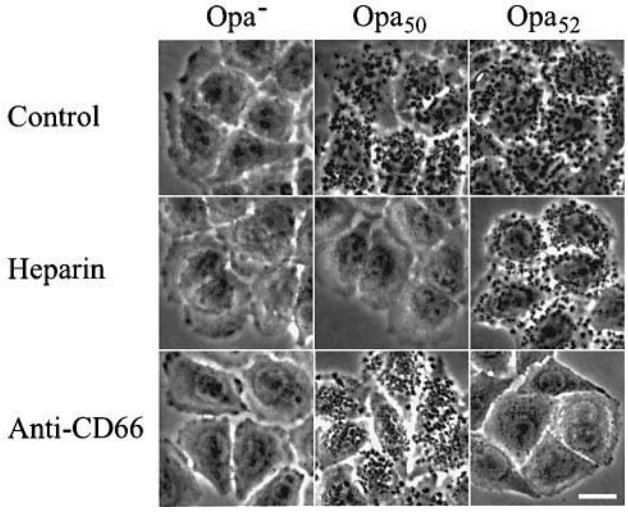

Fig. 4. Differential effects of heparin and CD66-reactive polyclonal antibodies on the Opa-mediated binding of *N.gonorrhoeae* strains to stably transfected HeLa cell lines expressing CGM1 (CD66d). The HeLa CGM1 cell line was infected with *N.gonorrhoeae* strains expressing either no Opa protein (Opa$^-$), the epithelial cell invasion-associated Opa protein known to interact with cell surface-associated proteoglycans (Opa$_{50}$) or an Opa protein mediating interaction with PMNs (Opa$_{52}$) for 3 h at 37°C and 5% CO_2 in RPMI 1640 medium containing no supplement (control), or supplemented with 100 μg/ml heparin or to 200 μg/ml of rabbit polyclonal anti-CEA antibodies (anti-CD66). After washing and fixing of the cells, phase-contrast pictures were taken. A scale bar corresponding to 10 μm is indicated in the lower right picture.

association with these individual cell lines are due to differences in binding affinity or receptor expression level is presently uncertain. Recombinant protein expression levels were assayed by Western blot analysis (data not shown), and confirmed that the absence of HeLa-CGM6 binding is not the result of a lack of CGM6 protein expression. Consistent with the receptor isolation studies (Figure 2), the absence of bacterial binding to HeLa-CGM6 supports the conclusion that Opa$_{52}$ does not recognize this CD66 family member.

In order to ascertain whether Opa$_{50}$ expression could allow binding to CD66 family members in addition to its previously described proteoglycan interactions (Chen *et al.*, 1995; van Putten and Paul, 1995), an adherence assay was done in the presence of heparin. In addition, a parallel assay was performed in the presence of polyclonal anti-CEA antiserum in order to determine what level of Opa binding could be attributed specifically to the CD66 receptors. Since the extracellular domain of CGM1 consists of only the conserved N-terminal domain common between all CEA family members, both competition assays were performed using the HeLa-CGM1 cell line. As is indicated in Figure 4, heparin almost completely abrogated Opa$_{50}$-mediated binding to HeLa-CGM1 but had no effect on the adherence of Opa$_{52}$-expressing gonococci. In contrast, the polyclonal antisera prevented binding by Opa$_{52}$- but not that of Opa$_{50}$-expressing strains to HeLa-CGM1 (Figure 4). Binding of Opa$_{52}$-expressing bacteria was also blocked when using HeLa-NCA, HeLa-BGP and HeLa-CEA (data not shown). Together, these assays indicate that Opa$_{50}$ and Opa$_{52}$ proteins do possess distinct specificities for either heparan sulfate-containing proteoglycans or CEA family antigens, respectively.

The distribution of CD66 antigen and bound gonococci on the cell surface was analysed by immunocytochemical staining and confocal laser scanning microscopy. As illustrated using the HeLa-CGM1 cell line in Figure 5, Opa$_{52}$-expressing gonococci appear to recruit CD66 family members to generate a focus of high receptor concentration at the site of bacterial adherence. Importantly, the absence of a similar phenomenon when Opa$_{50}$-expressing bacteria are employed confirms that this process is not a general cellular response to bacterial attachment. Parallel results obtained using the HeLa-NCA, HeLa-BGP and HeLa-CEA cell lines (data not shown) also indicate that the pattern of recruitment of their expressed CD66 antigens is indistinguishable from those depicted with HeLa-CGM1 in Figure 5, while no recruitment of CGM6 was evident when HeLa-CGM6 cells were infected with either Opa$_{50}$- or Opa$_{52}$-expressing gonococci (data not shown).

In order to analyse the potential for the CD66 family members to facilitate uptake of bound gonococci, a bactericidal concentration of gentamicin was added 3 h after the infection of cell lines to kill selectively extracellular gonococci (Makino *et al.*, 1991). Neither transparent nor Opa$_{50}$-expressing gonococci displayed any significant level of invasion under these assay conditions (Figure 6). Opa$_{50}$-mediated internalization by cultured HeLa cells is substantially enhanced by serum (O.Gómez-Duarte, M.Dehio, A.C.Guzmán, G.S.Chhatwal, C.Dehio and T.F.Meyer, in preparation), while uptake of Opa$_{52}$-expressing gonococci by the transfected cell lines expressing CD66 used in this study is not (data not shown). All adherence and invasion assays performed during this study were thus done in the absence of serum in order to avoid potential effects attributable to uncharacterized serum proteins and to highlight the difference between Opa$_{50}$- and Opa$_{52}$-mediated processes. Significant levels of cellular invasion occurred when the HeLa-CGM1, HeLa-BGP and HeLa-CEA cell lines were infected with Opa$_{52}$-expressing gonococci (Figure 6). Much lower numbers of intracellular bacteria were, however, recovered from the HeLa-NCA cell line, indicating that a strict correlation between binding (Figure 3) and invasion does not necessarily exist.

Opa$_{52}$ mediates binding and invasion of CD66-expressing HeLa cell lines by recombinant E.coli

The specific contribution of Opa proteins to the binding of CEA protein family members was assessed using recombinant *E.coli* containing the expression vector pTrc99a alone or expressing specific Opa proteins. Western blot analysis indicated that significant levels of Opa proteins were expressed by these recombinant strains (Kupsch *et al.*, 1993). Incubation of these recombinant strains on transfected HeLa cell monolayers displayed a pattern of binding reminiscent of that seen with Opa$_{50}$- and Opa$_{52}$-expressing gonococci. As is evident in Figure 7, *E.coli* expressing gonococcal Opa$_{50}$ bind equally well to HeLa-Neo and HeLa-BGP, while *E.coli* expressing Opa$_{52}$ adhere only to HeLa-BGP. Binding patterns on the other HeLa cell lines (data not shown) are also reminiscent of those seen for the respective gonococcal strains, thus verifying the central role of Opa in binding activities assayed throughout this study.

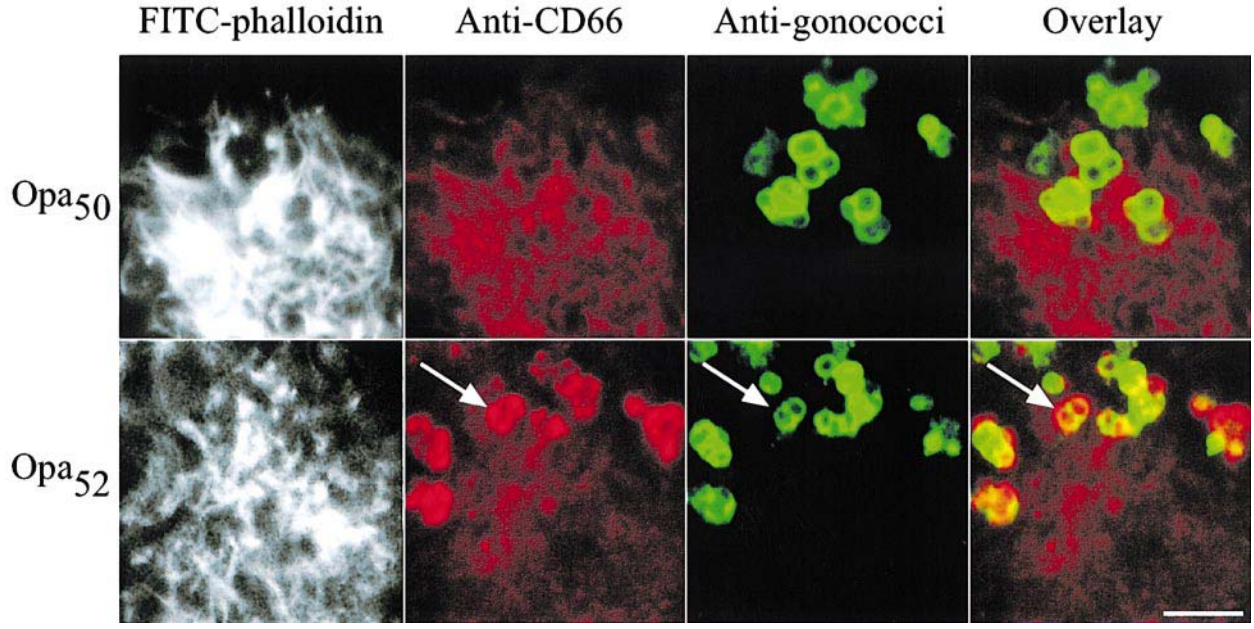

Fig. 5. Co-localization of Opa-expressing *N.gonorrhoeae* and CD66 on the surface of stably transfected HeLa cells expressing CGM1 (CD66d). The HeLa-CGM1 cell line was infected with *N.gonorrhoeae* strains expressing either the invasion-associated Opa protein mediating interactions with cell surface-associated heparan sulfates (Opa_{50}) or an Opa protein mediating interaction with PMNs (Opa_{52}) for 3 h at 37°C and 5% CO_2 in RPMI 1640 medium. Fixed samples were labelled for filamentous actin by FITC-labelled phalloidin, mAbs directed against CD66 (anti-CD66, red) and rabbit serum directed against gonoccoci (anti-gonococci, green). The corresponding pictures for the staining of CD66 and bacteria were superimposed (overlay) to monitor co-localization of CD66 to the vicinity of the bacteria interacting with the host cell surface, indicated by a yellow colour in the overlay. Arrows indicate the position of one representative bacterium associated with increased staining for CD66. A scale bar corresponding to 5 μm is indicated in the lower right picture.

In order to determine whether gonococcal factors other than Opa are required for CD66-mediated invasion of the transfected cell lines, gentamicin assays using the recombinant *E.coli* strains were performed. As is illustrated in Figure 8, the uptake of recombinant Opa_{52}-expressing *E.coli* by HeLa-BGP parallels that seen with *N.gonorrhoeae*, thus suggesting that no gonococcal factor other than Opa_{52} is necessary to mediate this internalization process.

Opa_{52} recruits CD66 antigens on human polymorphonuclear phagocytes

In order to correlate the distribution of CEA family members on granulocytic cells with that of associated gonococci, human PMNs were infected with gonococci for 1 h and the cells were then analysed by immunocytochemical staining methods and confocal laser scanning microscopy. Fixed samples were double labelled for CD66 antigens using mouse monoclonal antibody D14HD11 and for gonococci using polyclonal rabbit serum. Our preliminary studies indicated that, due to the large intracellular stores of CD66 residing in secondary and tertiary granules, a specific recruitment of CD66 to interacting Opa_{52}-expressing gonococci could not be demonstrated (data not shown). In order to reduce the background of CD66 staining resulting from these intracellular pools, we thus degranulated PMNs by PMA treatment prior to infection. Degranulation did not appear to have a significant effect on the number of cell-associated Opa_{50}- and Opa_{52}-expressing gonococci. Significantly, PMNs infected by Opa_{50}-expressing bacteria show a distribution of CD66 antigen over their cell surface (Figure 9) which is indistinguishable from that seen in uninfected cells (data not shown), while Opa_{52}-expressing bacteria cause a strong recruitment of CD66 proteins to the sites of interaction (Figure 9). Together, these observations support results obtained with the transfected HeLa cell lines which suggest that Opa_{52} mediates binding of CD66 antigens on PMNs while Opa_{50} does not.

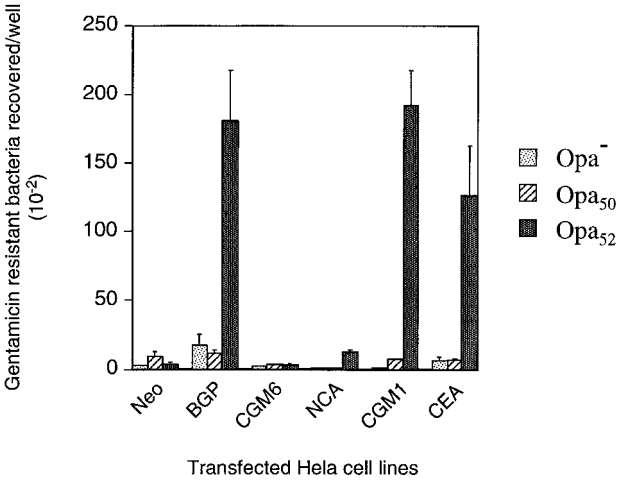

Fig. 6. Opa-mediated internalization of *N.gonorrhoeae* by CD66-expressing HeLa cell lines. *N.gonorrhoeae* expressing no Opa (Opa^-), the epithelial cell invasion-associated Opa_{50} or Opa_{52}, which mediates interactions with PMNs, were incubated with transfected HeLa cell lines expressing the indicated CEA family members as outlined in Materials and methods. Invasion assays were performed in triplicate on five separate occasions, and the data illustrated represent the mean results ± the standard deviation of one representative experiment.

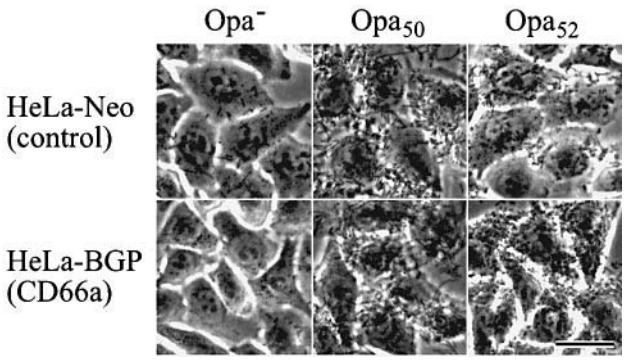

Fig. 7. Opa-mediated interactions of recombinant *E.coli* with stably transfected HeLa cells expressing BGP (CD66a). HeLa cell lines stably transfected with an empty vector control (Neo) or expressing CD66a (BGP) were infected with *E.coli* strains containing the expression vector pTrc99a alone (Opa^-), or expressing the epithelial cell invasion-associated Opa protein (Opa_{50}) or an Opa protein which mediates interaction with PMNs (Opa_{52}) for 3 h at 37°C and 5% CO_2 before washing and fixing. Phase-contrast pictures are presented. A scale bar corresponding to 20 μm is indicated in the lower right picture.

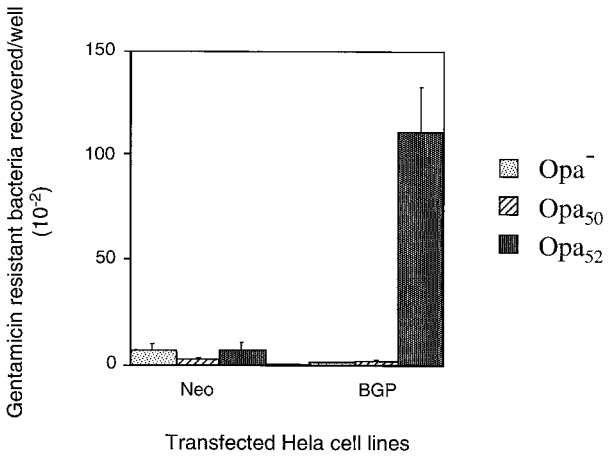

Fig. 8. Internalization of recombinant *E.coli* expressing gonococcal opacity protein by CD66-expressing HeLa cell lines. *E.coli* strains containing the expression vector alone (Opa^-) or expressing either the epithelial cell invasion-associated Opa protein (Opa_{50}) or an Opa protein mediating interaction with PMNs (Opa_{52}) were incubated with stably transfected HeLa cell lines containing the expression vector alone or expressing human BGP as outlined in Materials and methods. Invasion assays were performed in triplicate on four separate occasions, and the data illustrated represent the mean results ± the standard deviation of one representative experiment.

Discussion

Previously, gonococcal binding to human PMNs has been shown to be elevated following the PMA- or fMLP-stimulated release of secondary and tertiary granular contents into the extracellular milieu (Farrel and Rest, 1990). In addition, Shafer and Rest (1989) have indicated that outer membranes prepared from opaque gonococci are able to block the adherence of Opa-expressing *N.gonorrhoeae*, while membranes prepared from transparent (Opa^-) colonies cannot. Although these data do not demonstrate the presence of a saturable receptor being released during degranulation, they are consistent with such a hypothesis. In this study, a subset of the CD66 antigen-expressing CEA family, including CGM1, NCA and BGP, are shown to be cellular receptors bound by the gonococcal Opa proteins which mediate interactions with human PMNs. In addition, the epithelial cell-associated CEA protein was also seen to mediate Opa_{52} binding and gonococcal internalization by stably transfected HeLa cell lines. The strict specificities of Opa_{50} and Opa_{52} for proteoglycan and CEA family members, respectively, is particularly remarkable based upon the fact that these two gonococcal proteins share >71% identity over their entire sequence (Kupsch *et al.*, 1993). The importance of differences in protein sequence is, however, in itself an unreliable measure of functional similarity, as Opa_{60} is more homologous to Opa_{50} than it is to Opa_{52} (Kupsch *et al.*, 1993), yet Opa_{60}-expressing gonococci are indistinguishable from those which express Opa_{52} based upon all functional analyses presented in this study (data not shown). Consistent with the data presented here, Chen and Gotschlich (1996) recently have described the specific interaction between the chromosomal Opa_{52} homologue, OpaI, and CGM1, and Virji *et al.* (1996) have reported that three distinct Opa proteins expressed by *N.meningitidis* bind to BGP. Together, these results suggest that multiple Opa proteins expressed by both of the pathogenic Neisseriaceae may utilize this subset of CD66 antigens as a cellular receptor.

The CEA family of proteins belongs to the immunoglobulin superfamily, with members containing a single N-terminal IgV-like domain and a variable number of C2-type domains. In contrast to the other CEA family members, the extracellular portion of CGM1 consists of only a single IgV-homologous domain (Thompson *et al.*, 1991). Based upon gonococcal binding patterns presented here, Opa_{52} binding of the CD66 family members can therefore be localized to this 108 amino acid N-terminal domain of these molecules. Over this region, the level of similarity present between CEA and each of the other recombinant proteins employed in this study ranges between 71 and 89% (Thompson *et al.*, 1991). The pattern of gonococcal binding to transfected HeLa cell lines expressing CGM6-NCA chimeras indicates that the binding determinant resides within the first 59 amino acids at the N-terminus of the mature protein (C.Dehio, S.D.Gray-Owen, A.Haude, F.Grunert and T.F.Meyer, in preparation), a region which contains no glycosylation sites (Hefta *et al.*, 1990; Boehm *et al.*, 1996). This is consistent with our preliminary results which suggest that carbohydrate chains are not involved in Opa_{52}-mediated interactions with CD66 proteins.

Intriguingly, the binding mediated by Opa_{52} expression is not strictly correlated with the subsequent internalization of adherent gonococci by transfected HeLa cell lines expressing defined CD66 family members. Although interactions with NCA were detected by both biochemical (Figures 1 and 2) and *in vivo* binding studies (Figure 3), uptake of bound bacteria by HeLa-NCA is negligible in comparison with that which is seen with cell lines expressing CGM1, BGP or CEA (Figure 6). In neutrophils, the CD66 family members are associated with a tyrosine kinase activity which has been attributed to Lyn and Hck, both of which are members of the *src* family of protein kinases (Skubitz *et al.*, 1995). CGM1 and BGP possess transmembrane and intracellular domains in addition to their surface-exposed regions, while CEA, NCA and CGM6 are glycosyl phosphatidylinositol (GPI) anchored

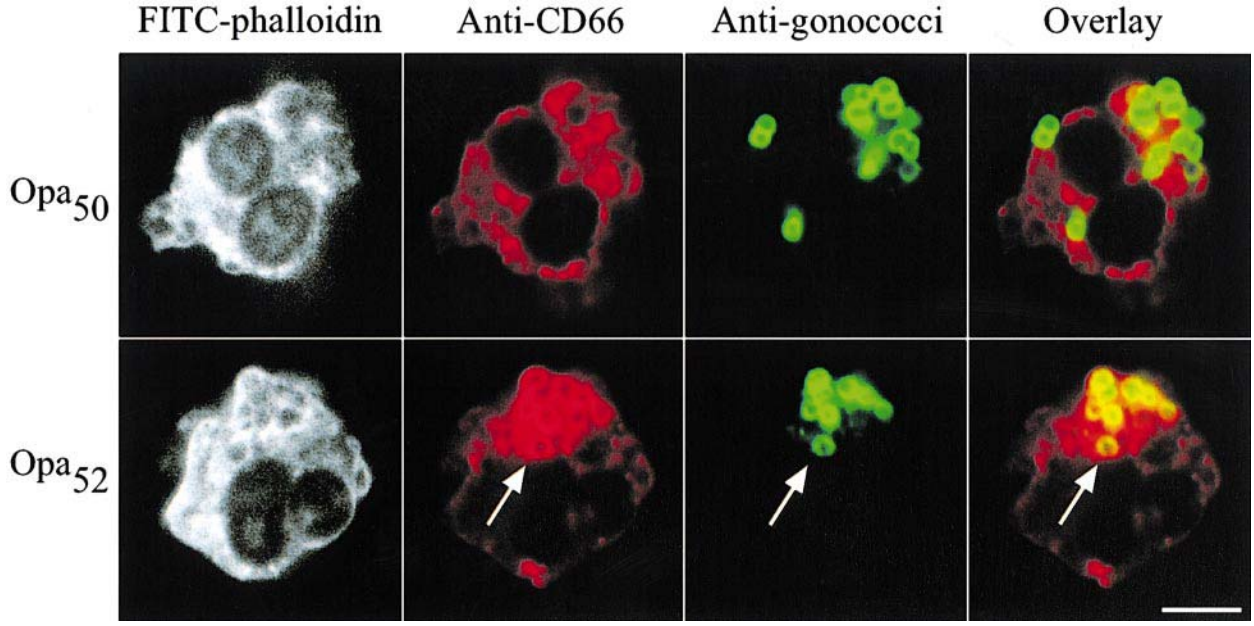

Fig. 9. Co-localization of CD66 antigens and Opa-expressing *N.gonorrhoeae* on the surface of human polymorphonuclear phagocytes. PMNs adherent to glass coverslips were degranulated with PMA and subsequently infected with *N.gonorrhoeae* strains expressing either the epithelial cell invasion-associated Opa protein which mediates interaction with cell surface-associated heparan sulfates (Opa_{50}), or expressing an Opa protein mediating interaction with PMNs (Opa_{52}) for 1 h at 37°C and 5% CO_2 in RPMI 1640 medium. Fixed samples were labelled for filamentous actin by FITC-labelled phalloidin, mAb D14HD11 cross-reactive for CD66a, c, d and e (anti-CD66, red) and rabbit serum AK93 directed against gonoccoci (anti-gonococci, green). The corresponding pictures for a staining of CD66 and bacteria were superimposed (overlay) to monitor co-localization of CD66 to the vicinity of bacteria interacting with the host cell surface, indicated by a yellow colour in the overlay. Arrows indicate one representative bacterium associated with increased staining for CD66. A scale bar corresponding to 5 μm is indicated in the lower right picture.

to the membrane. Whether differences seen in gonococcal internalization thus occur due to the distinct functional properties of NCA in general or due to a lack of expression of components necessary for an intracellular signalling cascade which initiates phagocytosis in the HeLa cell line are presently unknown. It is, however, interesting to speculate that mechanisms required for the epithelial cell-associated CEA protein's signal transduction may be present in the transfected HeLa cell lines used in this study while those required by the granulocytic NCA are not.

The distribution of a microbe's cellular receptors can delineate tissue tropism (Abraham *et al.*, 1983; Van de Water *et al.*, 1983; Flock *et al.*, 1987), and receptor function may determine whether binding leads to surface adhesion or internalization of the organism (Isberg and Tran Van Nhieu, 1994). *Neisseria gonorrhoeae* is able to express a repertoire of up to 11 distinct Opa proteins (Kupsch *et al.,* 1993). One of these variants mediates interactions with cell surface-associated proteoglycan molecules (Opa_{50}; Chen *et al.*, 1995; van Putten and Paul, 1995), while at least two others (Opa_{52} and Opa_{60}) are seen in this study to bind members of the CEA protein family which are expressed on granulocytes (CGM1, NCA, BGP) and colonic epithelia (CEA). The phase variability of Opa expression does, therefore, provide the gonococci with a varied tissue tropism without an associated loss of specialization. Phase transition of individual gonococcal *opa* loci is predicted to occur at a frequency of ~10^{-3} *in vitro* (Murphy *et al.*, 1989), thus resulting in the existence of ~1% of bacteria in a culture which are expressing one or more of the 11 variant Opa proteins (Bhat *et al.*, 1991). This is consistent with the finding that a gonococcal mutant containing an *opaC* deletion, and thus unable to express the epithelial cell-associated Opa_{50} homologue, is taken up by epithelial cells at a rate 5- to 10-fold lower than that seen with a visually selected transparent phase variant of the parental strain (Makino *et al.*, 1991; Kupsch *et al.*, 1993). Interestingly, whereas opaque variants originating from transparent gonococcal cultures *in vitro* typically expressed the chromosomally encoded homologue of Opa_{50}, nearly all bacteria recovered from male volunteers which had been intraurethrally inoculated with transparent gonococcal variants were opaque, and more than half of these expressed the Opa_{52} homologue (Swanson *et al.*, 1988). This suggests that Opa_{52}-mediated adherence to CD66 family members *in vivo* may facilitate the establishment and/or maintenance of infection. Interestingly, CEA-transgenic mice which in general maintain the correct spatio-temporal expression pattern of human CEA, do show its expression in urethra epithelia during development (Eades-Perner *et al.*, 1994). Whether it continues to be expressed at low levels in the adult urogenital tract or on other tissues which may be encountered by gonococci is, however, still uncertain. Similar conjectures have been made for the role of CEA as a receptor in the gastrointestinal tract for the type 1 fimbriae of *E.coli* and *Salmonella* which can mediate binding to D-mannosyl residues on CEA family members (Leusch *et al.*, 1991). This fact, along with the appearance of CEA protein in the colon after birth, has led to the speculation that CD66 members may function in the regulation of colonization by commensal bacteria (Leusch *et al.*, 1991). The CD66-mediated internalization of *E.coli* via fimbriae has not been reported, and the results of continuing studies in our laboratory appear to

confirm that gonococcal Opa_{52}-mediated binding occurs via a process which is mechanistically different from that of type 1 fimbriae.

Whether or not the massive recruitment of granulocytes to the urogenital tract is a sign of an effective immunological response to the organism is still undecided; however, the gonococci's ability to induce efficient phagocytosis in the absence of opsonins suggests that this process plays some role in facilitating either bacterial survival or transmission of this pathogen to other human hosts. PMN internalization of gonococci occurring via CD66-directed processes could potentially transport the bacteria into a niche which is protected from the bactericidal activities typical of opsonin-mediated phagocytosis. Unfortunately, although cross-linkage of CD66 members using specific antisera does result in an uptake of bound antibody (F.Grunert, unpublished observations), the processes which mediate this response or the intracellular processing which may occur after their internalization are still poorly understood. An interesting hypothesis for the benefits of Opa_{52} expression relies on the observation that these gonococci do not seem to induce a significant production of superoxide anion as measured by the oxidation of cytochrome *c* (Naids and Rest, 1991). Consistent with this observation is the relatively strong oxidative response elicited upon infection of phagocytic cells by commensal *Neisseria* species as compared with Opa_{52}-expressing gonococci (Hauck *et al*., 1997). If gonococci are truly capable of circumventing host cell oxidative responses via Opa_{52} expression, this might be due to an active process since Opa_{52}-expressing gonococci trigger an intracellular signal cascade which involves members of the Src family of kinases, while commensal *Neisseria* strains and Opa$^-$ gonococci do not (C.R.Hauck, T.F.Meyer, F.Lang and E.Gulbins, in preparation). This is consistent with the identification of the CEA family as receptors for Opa_{52} since CD66 proteins are associated with the Lyn and Hck kinases *in vivo* (Skubitz *et al.*, 1995). Together, these data assemble a tantalizing scenario in which *N.gonorrhoeae* appears to have evolved the means to penetrate to the interior of phagocytic cells while avoiding their bactericidal effects. In this context, the future unravelling of CD66 signalling and internalization pathways may provide important clues to the mechanisms involved in this process.

Materials and methods

Cell lines and bacterial strains

The construction of stably transfected HeLa cell lines expressing CEA (HeLa-CEA), CGM6 (HeLa-CGM6), NCA-90 (HeLa-NCA) or containing the expression vector alone (HeLa-Neo) were described by Berling *et al.* (1990). The construction of HeLa-CGM1 has also been described (Nagel *et al.*, 1993). HeLa-BGP was generated by cloning a full-length BGPa cDNA into the pRc-CMV vector, and transfection was performed as described previously (Nagel *et al.*, 1993). All HeLa cell lines were propagated in a humidified atmosphere at 37°C with 5% CO_2 in RPMI 1640 with L-glutamine (Life Technologies, Paisley, UK) supplemented with 10% fetal calf serum and 500 μg/ml geneticin.

The nomenclature used throughout this manuscript indicates the expressed recombinant gonococcal Opa protein as described by Kupsch *et al.* (1993). The pTrc99A expression vectors with or without appropriate recombinant *opa* genes employed in this study have been described previously (Kupsch *et al.*, 1993) except that *E.coli* strain JM103 was used as a host background. Gonococcal strain N279 is a non-piliated, S-pilin-expressing derivative of strain MS11 which carries a chloramphenicol resistance marker (*cat*) inserted into the chromosomal $opaC_{30}$ locus (Kupsch *et al.*, 1993). N303, N309 and N310 are derivatives of N279 which constitutively express recombinant Opa_{50}, Opa_{52} and Opa_{60}, respectively, which are not subject to phase variation typical of the chromosomal *opa* loci (Kupsch *et al.*, 1993). Transparent colonies of strain N302, which contains the expression vector without an associated *opa* coding sequence, was used as an Opa$^-$ control. All gonococcal strains were subcultured daily and were monitored routinely for their Opa phenotype using a binocular microscope upon passage. In addition, Opa expression was probed directly using immunoblot analysis of total bacterial extracts obtained from cultures used in infection and receptor isolation experiments.

Antibodies

Opa-specific monoclonal antibody 4b12/CII was generously provided by Dr Mark Achtman. Polyclonal anti-human CEA antiserum was purchased from DAKO (Glostrup, Denmark), the CGM6-specific monoclonal antibody 80H3 was purchased from Immunotech (Marseille, France) and the CD66 cross-reactive monoclonal antibody CLB/gran10 was purchased from Hiss Diagnostics (Amsterdam, The Netherlands). The characterization of all other anti-CD66 monoclonal antibodies has been described previously (Knapp *et al.*, 1989). Cy5-conjugated goat anti-rabbit antibodies were purchased from Jackson Immunoresearch Laboratories (Baltimore, MD) and Texas red-conjugated goat anti-mouse antibodies were purchased from Dianova (Hamburg, Germany).

Bacterial overlay assays

Detection of neutrophil components recognized by individual Opa-expressing bacterial strains was accomplished using a bacterial overlay of Western blotted proteins. Human PMNs were isolated from venous blood of healthy donors as described previously (Kupsch *et al.*, 1993). Recovered cells were diluted to a final concentration of 2×10^8 cells/ml and then diluted with an equal volume of 2× Laemmli sample buffer containing 1% 2-mercaptoethanol. Aliquots equivalent to 1×10^5 cells were separated by SDS–PAGE on a 4–15% gradient gel (BioRad Laboratories, München, Germany), electroblotted onto Immobilon-P transfer membrane and then blocked using a phosphate-buffered saline solution with 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$ (PBS/Mg/Ca) containing 5% skimmed milk powder.

Bacteria harvested from fresh overnight agar cultures were suspended to an OD_{550} of 1.0 in PBS/Mg/Ca, and NHS-LC-biotin (Pierce Biochemicals, Rockford, IL) was added to a final concentration of 250 μg/ml. The suspensions were incubated for 1 h at room temperature and then washed four times by centrifugation and resuspension in an equal volume of PBS/Mg/Ca. After washing, labelled bacteria were resuspended to an OD_{550} of 0.5 in PBS/Mg/Ca buffer containing 5% skimmed milk powder and then incubated overnight at 4°C with strips corresponding to individual lanes of the Western blotted PMN proteins. After washing three times with blocking solution, the filter strips were incubated with biotinylated SA-HRP (Sigma, Deisenhofen, Germany) and bound bacteria subsequently were detected using the chemiluminescent ECL detection kit (Amersham, Braunschweig, Germany).

Isolation of gonococcal Opa protein ligands from PMNs

The isolation of PMN components specifically recognized by individual gonococcal Opa protein variants was achieved by incubating intact bacteria with an extract prepared from labelled PMNs. The biotinylation of human PMNs was done based upon the method used by Isberg and Leong (1990) to label epithelial cells. Briefly, freshly isolated PMNs were resuspended to 2.5×10^7 cells/ml in PBS/Mg/Ca (pH 8.0), NHS-LC-biotin was added to obtain a final concentration of 500 μg/ml and the suspension incubated with gentle rocking for 30 min at room temperature. PMA was added to the biotinylation reaction and the mixture transferred to 37°C for a further 30 min. After centrifugation for 10 min at 550 *g*, the pellet was resuspended in fresh PBS/Mg/Ca (pH 8.0) to obtain 1×10^7 cells/ml. A 1:20 dilution of 1 M ethanolamine (pH 7.0) was added and the suspension was again incubated for 30 min at room temperature to quench remaining reactive biotin. After biotinylation, the cells were washed three times with PBS/Mg/Ca and the washed cell pellet was then resuspended in solubilization buffer consisting of PBS/Mg/Ca containing 2% Triton X-100, 10 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml pepstatin, 2.5 μg/ml leupeptin and 2 μg/ml aprotinin to obtain a cellular concentration of 10^8 PMNs/ml. After sonicating briefly to break up cell aggregates, the suspension was incubated for 1 h on ice. The Triton X-100-soluble fraction was recovered after centrifugation at 13 000 *g* for 5 min at 4°C in a microcentrifuge, and then diluted 1/10 in PBS/Mg/Ca buffer containing

1% bovine serum albumin (BSA) and 6 U/μl benzonase (Boehringer Mannheim, Germany). After 1 h, gonococci harvested from a fresh overnight culture were added to 1 ml of the benzonase-treated extract to a final OD_{550} of 1.0 and the suspension was incubated at 4°C for 2 h with gentle agitation. The bacteria were pelleted by centrifugation for 1 min at 4°C in a microcentrifuge and washed three times for 10 min with PBS/Mg/Ca buffer containing 0.2% Triton X-100 at 4°C with gentle agitation. The washed bacterial pellet was resuspended in 1 pellet volume of PBS, diluted 1:1 with 2× SDS–PAGE sample buffer containing 1% 2-mercaptoethanol and heated in a boiling water bath for 10 min prior to electrophoresis.

Infection of cells and transfected cell lines

For standard infection experiments, cells were seeded into 24-well cell culture plates to obtain cultures at ~70% confluence at the time of infection (~2×10^5 cells/well). Gonococci were harvested from fresh overnight cultures into RPMI 1640 medium with L-glutamine and 50 mM HEPES buffer (pH 7.3) to a culture density of OD_{550} = 0.1 and then incubated for 2 h at 37°C with shaking at 135 r.p.m.. For experiments involving *E.coli*, the bacteria were not pre-cultured in liquid medium. Prior to infection, cells were washed with 2×1 ml RPMI 1640 medium with L-glutamine. The wells were then inoculated with 1 ml of growth medium containing 2×10^7 bacteria/ml, thus yielding a bacteria/cell ratio of 100:1, and the gonococci were centrifuged onto the cell monolayer for 5 min at 500 r.p.m.. The centrifugation step was omitted when using recombinant *E.coli*. For antibody competition assays, 200 μg/ml anti-CEA IgG was added to the bacterial suspension. Prior to use, the azide was removed from the antiserum by replacement of antibody suspension buffer with RPMI medium using centrifugation through a Micron-30 microconcentrator with a molecular weight cut-off of 30 000 as described by the manufacturer (Amicon, Witten, Germany). After 3 h incubation at 37°C in an atmosphere containing 5% CO_2, the monolayers were washed three times with RPMI medium to remove non-adherent bacteria. For gentamicin assays, the level of internalized gonococci was ascertained by incubating the washed monolayers with RPMI medium containing 100 μg/ml gentamicin for 2 h to kill extracellular cell-associated bacteria. After washing to remove the antibiotic, 1 ml of 1% saponin in PBS/Mg/Ca was added per well and the plates were then incubated for 15 min at 37°C with 5% CO_2. After vigorous resuspension to release adherent and/or internalized bacteria from the lysed cells, appropriate dilutions were prepared and plated in order to quantify the remaining bacteria. In order instead to quantify total associated bacteria, gentamicin treatment was omitted and saponin lysis was therefore performed after the first washing steps.

For the infection of PMNs, freshly isolated cells were suspended in RPMI medium with L-glutamine (Gibco-BRL) and seeded onto glass coverslips in individual wells of a 24-well tissue culture plate to obtain a density of 1×10^6 cells/well. After 30 min incubation at 37°C in an atmosphere containing 5% CO_2 to allow cellular adherence to the solid support, degranulation was induced by the addition of PMA to a final concentration of 100 ng/ml. After 30 min, 3×10^7 bacteria were added per well and the plates were incubated for 1 h at 37°C. Coverslips were then washed three times with RPMI medium before fixation and immunocytochemical staining.

Immunocytochemistry, confocal laser scanning microscopy and phase contrast microscopy

For immunocytochemical analyses of infection experiments, cell monolayer preparation and infection were as outlined above, except that cells were initially seeded into wells containing 12 mm glass coverslips. Infections were stopped by washing three times with sterile PBS/Mg/Ca buffer, and cells were then fixed using 3.7% paraformaldehyde with 50 mM HEPES buffer for at least 20 min at room temperature. Triple stainings for CD66 antigens, bacteria and filamentous actin were performed by sequentially incubating the fixed cells with (i) 0.1% Triton X-100 in PBS for 15 min, (ii) 0.2% BSA in PBS for 15 min, (iii) mAb D14HD11 cross-reactive for CD66a, c, d and e diluted 1/100 in PBS for 1 h, (iv) Texas red-conjugated goat anti-mouse IgG antibodies diluted 1/100 in PBS for 1 h, (v) rabbit anti-*N.gonorrhoeae* antiserum (AK93) diluted 1/100 in PBS for 1 h, and (vi) a mixture of Cy5-conjugated goat anti-rabbit IgG antibodies diluted 1/100 and fluorescein isothiocyanate (FITC)-labelled phalloidin diluted to 1 μg/ml in PBS for 1 h. Three washings in PBS were done following each incubation step. Stained specimens were mounted in 0.1 M Tris, pH 8.5, 25% glycerol (w/v), 10% Moviol (w/v) and were viewed with a Leica TCS 4D confocal laser scanning microscope (Leica Lasertechnik, Heidelberg, Germany) equipped with an argon–krypton mixed gas laser. All three channels were recorded sequentially and the corresponding images were digitally processed with Photoshop 3.0 (Adobe Systems, Mountain View, CA). Phase-contrast pictures were taken by scanning fixed specimens with the transmission detector of the same confocal system connected to a Leica DMIRB inverted microscope.

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