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Differential Opa specificities for CD66 receptors influence tissue interactions and cellular response to *Neisseria gonorrhoeae*

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Summary

The ability of all 11 variable opacity (Opa) proteins encoded by *Neisseria gonorrhoeae* MS11 to interact directly with the five CD66 antigens was determined. Transfected HeLa cell lines expressing individual CD66 antigens were infected with recombinant *N. gonorrhoeae* and *Escherichia coli* strains expressing defined Opas. Based upon the ability of these bacteria to bind and invade and to isolate specifically CD66 antigens from detergent-soluble extracts of the corresponding cell lines, distinct specificity groups of Opa interaction with CD66 were seen. Defining these specificity groups allowed us to assign a specific function for CD66a in the Opa-mediated interaction of gonococci with two different target cell types, which are both known to co-express multiple CD66 antigens. The competence of individual Opas to interact with CD66a was strictly correlated with their ability to induce an oxidative response by polymorphonuclear neutrophils. The same Opa specificity was observed for the level of gonococcal binding to primary endothelial cells after stimulation with TNFα, which was shown to increase the expression of CD66a rather than CD66e. As CD66e alone is expressed on other target tissues of gonococcal pathogenicity, Opa variation probably contributes to the cell tropism displayed by gonococci.

Introduction

Initial attachment of *Neisseria gonorrhoeae* to human urogenital mucosa is mediated by the pilus (McGee *et al.*, 1983; Rudel *et al.*, 1992). However, a tight secondary association with epithelial tissues is conferred by expression of the phase variable- and colony opacity-associated Opa outer membrane proteins. Although several of the 11 distinct Opa proteins expressed by *N. gonorrhoeae* MS11 are associated with an increased level of binding to cultured epithelial cell lines, only one of these proteins confers an invasive phenotype (Kupsch *et al.*, 1993). This entry is mediated by heparan sulphated proteoglycan (HSPG) receptors expressed on the surface of target cells (Chen *et al.*, 1995; van Putten and Paul, 1995) and is significantly enhanced in some cell lines by the presence of serum-derived vitronectin (Duensing and van Putten, 1997; Gomez-Duarte *et al.*, 1997). Whether this internalization process is analogous to the transcytotic penetration of epithelial cells by gonococci in fallopian tube organ cultures is still uncertain (Ward *et al.*, 1974; McGee *et al.*, 1981), yet the existence of disseminated gonococcal disease (Handsfield, 1975) confirms this bacteria's ability to enter the underlying tissues *in vivo*.

Gonorrhoea is associated with an intense inflammatory response involving leucocytic infiltrations of the infected urogenital mucosa. The efficient binding and non-opsonic phagocytosis of *N. gonorrhoeae* by polymorphonuclear neutrophils (PMNs) and monocytes is dependent upon the expression of specific Opa proteins (King and Swanson, 1978; Virji and Heckels, 1986; Fischer and Rest, 1988), as is the enhanced respiratory burst, which signals the bactericidal response of these professional phagocytes (Naids and Rest, 1991; Belland *et al.*, 1992). Recently, we and others have reported that the closely related CD66a (biliary glycoprotein; BGP), CD66c (non-specific cross-reacting antigen; NCA) and CD66d (CEA gene family member 1; CGM1) can function as neisserial Opa receptors on PMNs, whereas CD66b (CGM6) does not (Chen and Gotschlich, 1996; Virji *et al.*, 1996a; 1996b; Gray-Owen *et al.*, 1997). Individual members of the CD66 family have different tissue distributions (Berling *et al.*, 1990; Thompson *et al.*, 1991; Prall *et al.*, 1996), suggesting that Opa-mediated interactions with non-granulocytic cell types could also be mediated via CD66 binding. Consistent with this premise, a gonococcal Opa variant was also shown to mediate adherence to, and invasion of, stably transfected HeLa cell lines expressing CD66e (carcinoembryonic

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antigen; CEA) (Chen *et al*., 1997; Gray-Owen *et al*., 1997), a related protein which is absent from granulocytes but is expressed on certain epithelial and endothelial tissues (Majuri *et al*., 1994; Prall *et al*., 1996).

Recently, Bos *et al*. (1997) used *in vitro* infection assays to measure gonococcal interactions with stably transfected CD66-expressing cell lines. Using this technique, they reported four different Opa proteins specifically conferring binding to CD66c- and CD66d-expressing cells, while all Opa-expressing gonococci bound to cell lines expressing either CD66a or CD66e. In the present study, we have used a combination of infection assays and a receptor isolation strategy using both *E. coli* and *N. gonorrhoeae* strains, which express each of the defined recombinant gonococcal Opas to show that differential specificities do exist for the CD66a and CD66e receptors. The significance of these findings is supported by *in vitro* studies showing an Opa-dependent adherence to CD66a expressed by human umbilical vein endothelial cells (HUVECs) after tumour necrosis factor alpha (TNFα) treatment, and by the specific correlation between CD66a binding and the induction of an oxidative response by human PMNs.

Results

Distinct gonococcal MS11 Opa proteins mediate different patterns of interaction with transfected HeLa cell lines expressing recombinant CD66 receptor proteins

Previously, we have shown that expression of Opa_{52} by recombinant *E. coli* and gonococcal strains confers the ability to attach and enter the stably transfected HeLa cell lines expressing either CD66a, c, d or e, but not those expressing CD66b (Gray-Owen *et al*., 1997). Consistent with this data, Chen and Gotschlich (1996) identified CD66d as a receptor for the Opa_{60} homologue, OpaI, and Virji *et al*. (1996a) showed that CD66a interacts with meningococcal Opa proteins. In order to ascertain which of the 11 genetically defined *N. gonorrhoeae* MS11 Opa protein variants can mediate cellular interactions via the CD66 proteins, CD66-expressing HeLa cell lines were infected with recombinant *E. coli* DH5 strains expressing individual Opa proteins or containing the Hermes-10 shuttle vector alone. The use of recombinant *E. coli* in this study allowed the specific contribution of Opa proteins for receptor binding to be determined in this heterologous background. In addition, it precluded the possibility that binding detected could be caused by the expression of chromosomally encoded Opa proteins, as their phase variability typically results in heterologous Opa variants being expressed by approximately 1% of a gonococcal population (Bhat *et al*., 1991). Bacterial association with these cell lines was determined by quantifying colony-forming units recovered after saponin lysis of the cellular membranes. Except for Opa_{50}, which mediates interactions with HSPGs, all other *N. gonorrhoeae* MS11 Opas confer CD66 receptor binding on the recombinant *E. coli* strains. However, four different CD66 specificity patterns exist (Fig. 1), indicating that these Opa–CD66 binding interactions are not strictly conserved. Opa binding of CD66 receptors typically correlates with bacterial internalization by the respective cell lines, as indicated by gentamicin survival assays, the only exception being that the Opa_{60}-expressing strain invades HeLa-CD66c only at a very low level (Fig. 1). Only Opa_{50} conferred any significant binding to either HeLa-Neo, which contains the empty expression vector, or HeLa-CD66b (data not shown). FACS analysis to quantitate CD66 expression levels of the transfected cell lines confirmed that there is no correlation between differences in the cell lines' receptor density and either bacterial adherence or invasion (data not shown). Significantly, adherence corresponded to up to a 20-fold higher recovery of intracellular bacteria from cell lines expressing CD66d compared with those recovered from the other CD66-expressing HeLa cells.

To confirm that binding seen with the Opa-expressing *E. coli* strains correlated with the expressed Opas' ability to mediate gonococcal binding, recombinant *N. gonorrhoeae* strains constitutively expressing defined Opa proteins (Kupsch *et al*., 1993) were used in parallel infection experiments. As shown in Table 1, the binding pattern of recombinant gonococci to HeLa cells expressing either CD66b, c, d or e was generally consistent with that seen when using the *E. coli* strains, except that Opa_{60}-expressing gonococci interacted with HeLa-CD66d (Table 1), while *E. coli* expressing this protein does not (Fig. 1). The reason for this difference is uncertain. However, the Opa_{60}-mediated binding and invasion seen with HeLa-CD66a, c and e does indicate that this protein is functional in *E. coli* (Fig. 1). Also, gonococcal association with HeLa-CD66a cells occurred irrespective of Opa expression (compare Fig. 1 and Table 1). The recipient *N. gonorrhoeae* MS11 strain N279 contains a deletion in the chromosomal *opaC* locus encoding the epithelial cell adherence and invasion-associated Opa_{30} protein, but the other 10 chromosomal *opa* loci are still intact, and their expression can be turned on by phase variation (Meyer and van Putten, 1989). Since transparent (Opa^-) gonococci also displayed a low level of binding to CD66a-expressing HeLa cells, adherent bacteria remaining after infection of these cell lines with strain N302 Opa^- were recovered by saponin lysis of the HeLa cell membranes. Both colony morphology and immunoblot analysis of recovered bacteria indicated that this adherence was not mediated by Opa (data not shown). Previous work has shown CD66-specific antibodies to inhibit binding of defined neisserial (Virji *et al*., 1996a; Chen and Gotschlich, 1996; Gray-Owen *et al*.,

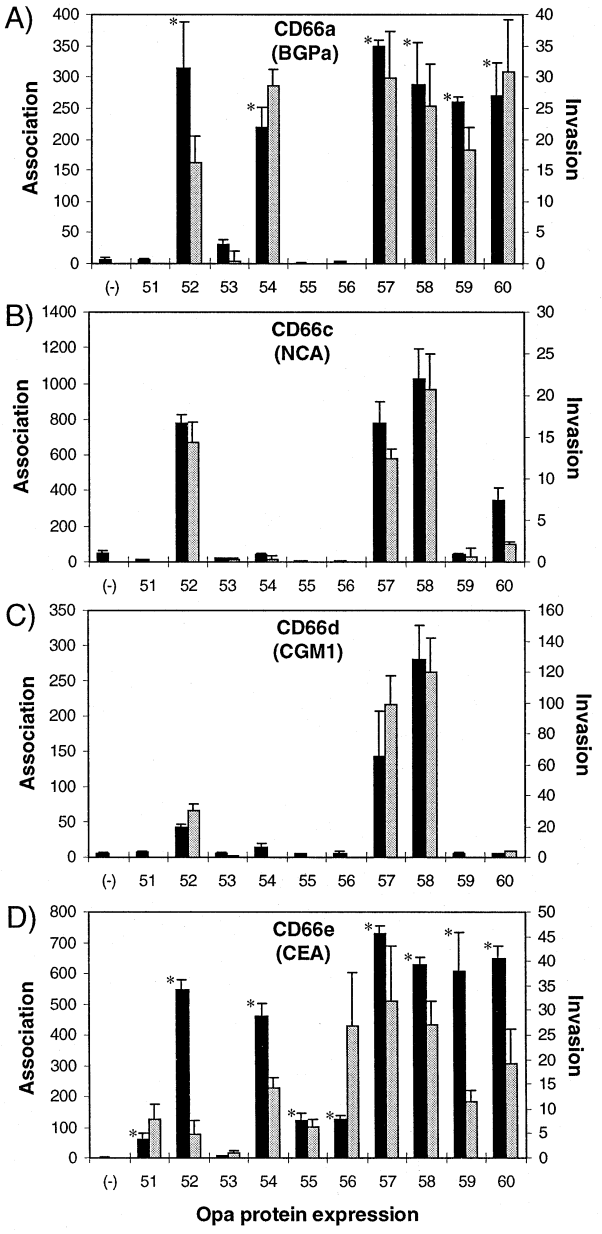

Fig. 1. Opa-mediated interactions with stably transfected CD66-expressing HeLa cell lines.
A. HeLa-CD66a.
B. HeLa-CD66c.
C. HeLa-CD66d.
D. HeLa-CD66e. These cell lines were infected for 5 h with *E. coli* strains expressing defined recombinant Opa proteins, viable bacteria were quantified by lysis of cellular membranes directly (associated, black bars) or after gentamicin treatment (invasion, grey bars). Scales for associated and intracellular bacteria recovered are different for each cell line in order to display significant differences between Opa-mediated interactions. Assays were performed in triplicate on at least two separate occasions, and data illustrated represent the mean ($\times 10^{-4}$) ± the standard deviation of one representative experiment. Asterisks indicate binding interactions that have been confirmed by receptor isolation experiments (see Fig. 2).

1997) Opas to cell lines expressing CD66a and d respectively. Here, we have expanded this work to show that anti-CD66 antibodies inhibit all Opa-mediated interactions except those by Opa_{50}-expressing gonococci (Table 1), confirming that even Opa^- interactions are a result of specific interactions with expressed CD66 receptors. Conversely, heparin only inhibited binding mediated by the heparan sulphate proteoglycan (HSPG)-specific Opa_{50} (Table 1).

In order to determine if the Opa-independent gonococcal binding to HeLa-CD66a might be an artifact specific for HeLa cells, CHO cells were instead transfected with a pRC/CMV-CD66a expression construct. In addition, we also transfected the mutant cell line, CHO-*pgs*677, which is defective in heparan sulphate synthesis and therefore does not mediate binding by Opa_{50}, in order to test if Opa_{50} may also have the capacity to bind to CD66 receptors. Subsequent infection of these transiently transfected cells clearly demonstrated that N302 Opa^- did not bind to CD66a-expressing cells from either CHO-K1 or CHO-*pgs*677. Whether the differences seen in Opa-independent interactions with the transfected HeLa and CHO cell lines are related to a previously reported difference in CD66a epitope expression by these cell lines (Jantscheff *et al.*, 1996) or result from uncharacterized differences in expression and/or processing of CD66a is still uncertain. N303 Opa_{50} bound to CHO-K1 regardless of its CD66 expression but did not bind any CHO-*pgs*677 cells, confirming that it does not also bind CD66 receptors. Gonococci expressing other Opa proteins showed a pattern of binding that is more consistent with the *E. coli* data (Fig. 1), although some binding by the strains expressing Opa_{51}, Opa_{55} and Opa_{56} does suggest that they may be able to mediate a low level binding of CD66a (Table 1). Together, this data suggests that HSPG and CD66 receptors are distinct binding interactions conferred by Opa_{50} or the other 10 Opa proteins, respectively, and that a different spectrum of CD66 proteins may be recognized by individual Opa proteins.

Opa-mediated interactions correlate with the isolation of CD66 receptor proteins from transfected HeLa cell lines

Initially, we used an affinity isolation assay system employing whole gonococci constitutively expressing recombinant Opa proteins to identify CD66a, c and d as the Opa_{52} receptors on human PMNs (Gray-Owen *et al.*, 1997). In order to confirm the differential Opa-binding specificities for CD66a and e, a similar strategy was used to isolate specifically receptor proteins from the stably transfected HeLa cell lines. As each cell line expresses only one CD66 family member, this approach avoided the possibility that a higher affinity for one of the CD66 receptors would prevent detection of Opa-mediated interactions occurring with the

Table 1. Adherence of recombinant gonococci to transfected cell lines expressing defined CD66 family members.

		HeLa*																		CHO-K1	CHO-*pgs*677
		Neo			CD66a			CD66b			CD66c			CD66d			CD66e			CD66a	CD66a
Strain	Opa	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	a
N302	–	–	–	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
N303	50	+	–	+	+	+	+	+	–	+	+	–	+	+	–	+	+	–	+	+	–
N305	51	–	–	–	++	++	–	–	–	–	–	–	–	–	–	–	++	++	–	+	+
N309	52	–	–	–	++	++	–	–	–	–	++	++	–	++	++	–	++	++	–	++	++
N304	53	–	–	–	++	++	–	–	–	–	–	–	–	–	–	–	+	+	–	+	+
N311	54	–	–	–	++	++	–	–	–	–	–	–	–	–	–	–	++	++	–	++	++
N307	55	–	–	–	++	++	–	–	–	–	–	–	–	–	–	–	++	++	–	+	+
N308	56	–	–	–	++	++	–	–	–	–	–	–	–	–	–	–	++	++	–	+	+
N313	57	–	–	–	++	++	–	–	–	–	++	++	–	++	++	–	++	++	–	++	++
N312	58	–	–	–	++	++	–	–	–	–	++	++	–	++	++	–	++	++	–	++	++
N306	59	–	–	–	++	++	–	–	–	–	–	–	–	–	–	–	++	++	–	++	++
N310	60	–	–	–	++	++	–	–	–	–	++	++	–	+	+	–	++	++	–	++	++

* Binding was assayed in RPMI-1640 medium supplemented with: (a) unsupplemented; (b) 100 μg ml^{-1} heparin; or (c) 100 μg ml^{-1} polyclonal anti-CEA IgG (Dako). (–) no binding; (+) binding; (++) strong binding.

other CD66 proteins expressed by PMNs. It also allowed the examination of Opa-mediated interactions with CD66e, as this protein is not expressed by PMNs. As evident in Fig. 2, the recovery of CD66a and e by recombinant Opa-expressing *E. coli* strains correlates well with their ability to mediate adherence to and invasion of the respective transfected cell lines (Fig. 1, asterisks). As a control, the Opa expression of recombinant *E. coli* strains is shown (Fig. 2D). Importantly, similar results were obtained when recombinant gonococcal strains constitutively expressing the same Opa proteins were used instead (data not shown). This confirms that the pattern of CD66 protein binding detected when using Opa-expressing *E. coli* strains represents the specific interactions that occur between Opa and the CD66 receptors expressed by transfected epithelial cells.

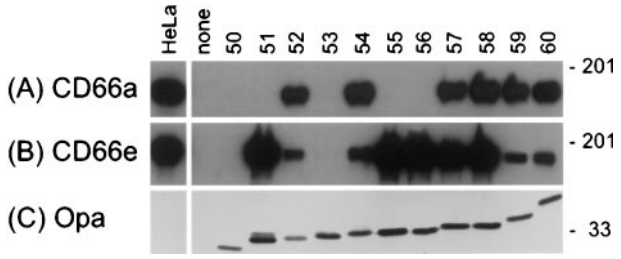

Fig. 2. Opa-mediated isolation of CD66 antigens from stably transfected HeLa cell lines. Triton X-100 extracts prepared from HeLa cell lines expressing CD66a (A) or CD66e (B) were incubated with *E. coli* strains expressing recombinant gonococcal Opa proteins. Component proteins from the HeLa extract (HeLa) or associated with the washed bacterial pellet were separated by SDS–PAGE, electroblotted and then probed using the CD66-specific CLB/gran10 monoclonal antibody. No CD66 antigens were isolated from the HeLa-Neo cell line, which contains the expression vector alone (data not shown). A replicate blot was probed with the anti-Opa (C) mAb, 4B12C11 (Achtman *et al*., 1988), in order to confirm Opa expression patterns and compare protein levels applied per lane.

Induction of oxidative burst by human PMNs correlates with Opa binding specificity for CD66a

Although the gonococcal opacity phenotype typically correlates with an increased level of interactions with PMNs (King and Swanson, 1978; Virji and Heckels, 1986; Fischer and Rest, 1988), an enhanced oxidative response does not always occur (Fischer and Rest, 1988). The production of reactive oxygen metabolites in response to specific stimuli can be quantified by the measurement of luminol-enhanced chemiluminescence (CL; Rest and Speert, 1994). Previously, Belland *et al*. (1992) used recombinant strains expressing a limited set of gonococcal Opa proteins to show that only some Opas could induce a PMN oxidative response. The availability of a complete set of recombinant *E. coli* and *N. gonorrhoeae* strains expressing all gonococcal MS11 Opa variants allowed us to correlate CD66 receptor specificity with CL induction directly. As is shown in Fig. 3, the respiratory burst of PMNs infected with gonococcal strains was stimulated strongly by expression of either Opa_{52}, Opa_{54}, Opa_{57}, Opa_{58}, Opa_{59} or Opa_{60}, and to a much lower extent also by Opa_{53}. Significantly, this result strictly correlates with the ability of each Opa to bind CD66a (Fig. 1). Since this CL pattern was also seen when using recombinant Opa-expressing *E. coli* strains (Fig. 3), these results suggest that the Opa-mediated binding of CD66a on PMNs is sufficient to induce a CL response by itself.

The level of gonococcal adherence to TNFα-treated HUVECs correlates with the binding capacity of expressed Opas for CD66a

During disseminated infection, gonococci are exposed to endothelial tissues (Handsfield, 1975). Endothelial cells have been reported to express both CD66a and CD66e

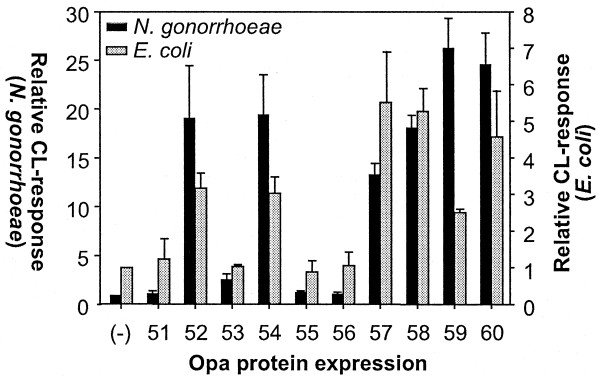

Fig. 3. Opa-mediated stimulation of oxidative metabolism by infected polymorphonuclear phagocytes. Freshly isolated PMNs were incubated in the presence of *N. gonorrhoeae* or *E. coli* expressing defined recombinant Opa proteins at a multiplicity of infection of 20 bacteria/cell. The oxidative response of PMNs was quantified by luminol-enhanced chemiluminescence, and results are expressed as the integral sum of total counts generated over 60 min compared with that stimulated by Opa-negative strains. Data illustrated represent the mean results ± the standard deviation of three independent experiments using blood from different donors.

(Majuri *et al.*, 1994; Prall *et al.*, 1996), with their expression being upregulated by proinflammatory cytokines such as TNF-α or IFN-γ (Majuri *et al.*, 1994). In order to examine the significance of CD66 binding for adherence to endothelial cells *in vivo*, primary endothelial cells were isolated from human umbilical veins and then infected with gonococcal strains expressing defined Opa proteins (Fig. 4). Quantitation of bacterial adherence by confocal microscopy indicates that both the HSPG-specific Opa_{50} and CD66a-specific Opa proteins can mediate binding to untreated HUVECs. However, a significant increase in bacterial association upon TNF-α treatment occurred only when CD66a-specific Opas are expressed (Fig. 4B). In all cases, only a portion of the cells bound bacteria (Fig. 4A). This is consistent with FACS analysis of CD66 expression patterns using the CD66 cross-reactive mAb D14HD11 (Fig. 4C), which indicates that after stimulation these primary cells display a heterogeneous pattern of CD66 expression. In contrast to the elevated binding of mAb D14HD11 after stimulation with TNFα, no significant differences are associated with the CD66e-reactive antibody, mAb 26/3/13 (Fig. 4C). The increase in gonococcal adherence following TNFα treatment and a binding pattern of the various Opas that is consistent with their specificity for CD66a suggests that CD66a expression is upregulated and may be the primary CD66 receptor for gonococcal Opas on endothelial cells during inflammation.

Discussion

CD66 antigens belong to the carcinoembryonic antigen (CEA) family of the immunoglobulin superfamily. CD66a and d possess a C-terminal transmembrane sequence and a short intracellular domain, while the others are linked to the cellular membrane via a glycosyl phosphatidyl-inositol (GPI) anchor. The highly conserved N-terminus of CEA family members shows homology to the immunoglobulin variable domain and is typically followed by a variable number of immunoglobulin constant domain (Ig_C)-like sequences (Thompson *et al.*, 1991). Since the 30 kDa CD66d receptor protein lacks any Ig_C-homologous domain, structure(s) recognized by Opa must occur within the N-terminal 108 amino acids of these receptors. Consistent

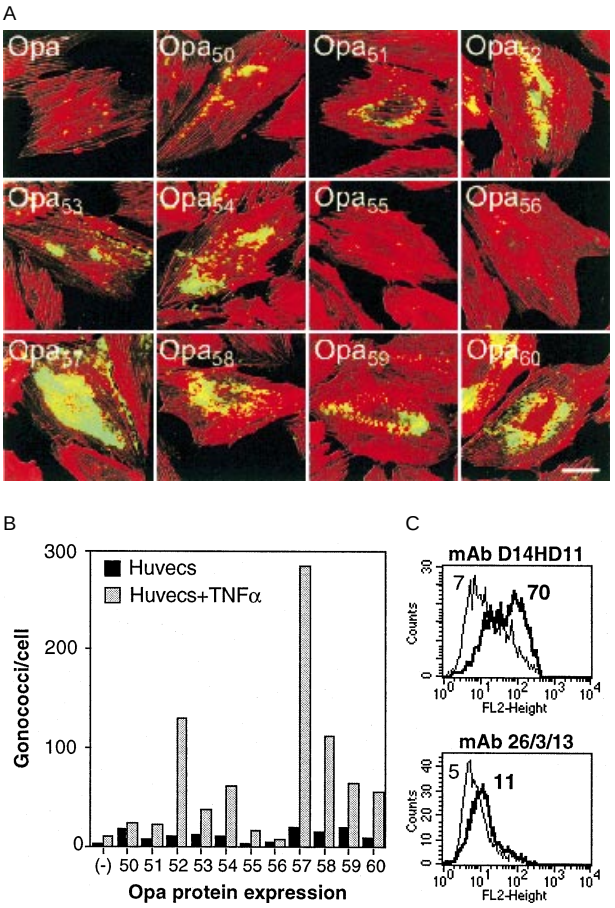

Fig. 4. Gonococcal association with endothelial cells correlates with CD66 expression. HUVECs with or without stimulation with the inflammatory cytokine TNFα were infected with gonococci expressing defined Opa proteins, and their adherence to individual cells was quantified by confocal microscopy.
A. Fixed samples of TNFα-treated HUVECs were stained for F-actin with FITC-labelled phalloidin (red label) and anti-gonococcal antiserum and secondary Texas red-conjugated antibodies (green label, appears yellow when co-localizing with F-actin staining in red) to visualize bacterial binding.
B. Mean number of bacteria associated per cell ($n = 30$) for HUVECs with or without TNFα treatment. Data presented represent one of two independent experiments.
C. FACS analysis of CD66 expression by HUVECs cells with or without TNFα treatment using CD66a, c, d and e-specific mAb D14HD11 and CD66e-specific 26/3/13. Numbers in graph indicate peak channel fluorescence for each sample.

with this, Virji *et al.* (1996a; 1996b) used soluble chimeric receptor proteins to show that the CD66a N-terminal domain is sufficient to mediate interactions with meningococcal Opas. Interestingly, this region is over 70% identical among the various CD66 proteins, yet CD66b is not recognized by any of the gonococcal Opa proteins.

The function of CD66 proteins *in vivo* is still uncertain. However, their ability to mediate both homotypic and heterotypic interactions suggests a role in intercellular adhesion (Oikawa *et al.*, 1991; Kuijpers *et al.*, 1992). Non-stimulated granulocytes express relatively low levels of CD66a, b, c and d at their surface, but neutrophil stimulation and subsequent degranulation results in the upregulation of CD66a, b and c surface expression (Kuijpers *et al.*, 1991). CD66a and c are involved in neutrophil adherence to inflammatory TNF-α or IL-1 cytokine-activated endothelial cells, both directly through their ability to present the sialylated Lewis^x antigen to E-selectin and indirectly by the CD66c-stimulated activation of CD18 integrins (Kuijpers *et al.*, 1992). It is interesting in this respect that gonococcal infection induces the synthesis of TNF-α and IL-1β transcripts in epithelial cells (Naumann *et al.*, 1997), as a similar phenomenon occurring in endothelial tissues would result in the recruitment of granulocytes to the infected site. We have also seen that TNF-α treatment of HUVECs significantly increases expression of CD66a, as measured by FACS analysis and CD66a-specific binding by gonococcal Opas (Fig. 4). Thus, one may speculate that gonococcal infection of endothelia *in vivo* could trigger the increased synthesis of CD66a as a gonococcal Opa receptor by stimulating the autocrine synthesis of TNF-α.

Consistent with our results, Bos *et al.* (1997) reported previously that Opa$^-$ gonococci also interact with certain CD66-expressing cell lines. These interactions are of a different nature from the Opa-mediated binding, as the former do not mediate significant invasion (Bos *et al.*, 1997) and do not allow isolation of either CD66a or CD66e from extracts prepared from the transfected cell lines (Fig. 2). *N. gonorrhoeae* can express multiple factors that contribute to cellular adherence (Jerse and Rest, 1997), including an unidentified adhesin, which mediates binding to lactose-containing carbohydrate structures (Stromberg *et al.*, 1988). How each of these factors contribute to interactions with the highly glycosylated CD66 molecules is still uncertain. The use of recombinant *E. coli*-expressing defined gonococcal Opas (Fig. 1) and the direct isolation of CD66 proteins from transfected cell extracts (Fig. 2) has allowed us to characterize the specific Opa-CD66 interactions better, a goal that is more difficult to assess when using gonococcal strains alone (Bos *et al.*, 1997; Table 1). The different Opa specificities for CD66a and CD66e are significant in view of the fact that only CD66a-specific Opas (Figs 1 and 2) are capable of inducing a granulocytic CL response (Fig. 3) and of colonizing endothelial tissues *in vitro* (Fig. 4). Although gonococci do demonstrate some potential to bind HeLa-CD66a-expressing strains, a high binding avidity for CD66a may be particularly important for granulocytic interactions, which lead to the oxidative response, as resting neutrophils express less than 5% of the CD66a receptor/cell that is present on the transfected cell lines used in this and other studies (Bos *et al.*, 1997; Chen *et al.*, 1997).

Differential Opa–CD66 interactions also have important implications for tissue tropism and disease manifestations of gonococcal infection. CD66e is expressed on squamous epithelium of the uterine portio, while CD66a is expressed on epithelium of the cervix, uterus and colon (Prall *et al.*, 1996). Transgenic mice, which generally maintain the same spatiotemporal CD66e expression patterns as humans, also express this protein in the urethra and rectum (Eades-Perner *et al.*, 1994), both of which are colonized during gonococcal infection of humans. Disseminated gonococcal infection (DGI) may be influenced by the presence of CD66a on the endothelium of vessels in the endometrium (Prall *et al.*, 1996) and CD66e on endothelium in lymph nodes and inflammatory tissues (Majuri *et al.*, 1994). The expression of CD66a on uterine glands, which span the entire extent and thickness of the endometrium and become enlarged during the premenstrual period (Prall *et al.*, 1996), is also intriguing, as the appearance of pelvic inflammatory disease and disseminated gonococcal infection are both related to the onset of menstruation (Holmes *et al.*, 1971; Sweet *et al.*, 1981). As CD66 and HSPG receptors may be co-expressed on various tissues, their relative contribution to the infectious process still remains to be elucidated. However, our results suggest that the differential expression of CD66-specific Opa variants can mediate the colonization of those tissues that are encountered during gonococcal infection.

Although it is clear that certain Opas can stimulate the induction of an oxidative response by phagocytic cells (Fig. 3; Belland *et al.*, 1992), it is still uncertain as to why gonococci would express an adhesin capable of inducing what is normally a bactericidal response. Virulent gonococci stimulate the release of secondary but not primary granules (Densen and Mandell, 1978; Farrel and Rest, 1990), an observation that is consistent with the result of CD66 cross-linking studies using antibodies *in vitro* (Klein *et al.*, 1996). Interestingly, degranulation only occurs when antibodies cross-reactive for CD66a, c and d are used, thus suggesting that the cross-linking of all three CD66 antigens is required for this effect (Klein *et al.*, 1996; Stocks *et al.*, 1996). The induction of a CL response by Opa_{54} and Opa_{59}, both of which we show here to bind CD66a on PMNs exclusively, suggests that this is not the case. Stocks *et al.* (1996) have proposed that CD66a, b and c are associated together as a complex on the neutrophil cell surface. If true, our results suggest that the

induction of a respiratory burst may instead require the cross-linking of CD66 receptor complexes. Such an event may not be possible if the antibodies used are unable to span the distance between CD66a molecules of adjacent receptor complexes, whereas the high density of Opa proteins expressed on the bacterial surfaces could.

The cytoplasmic domain of CD66d has a sequence reminiscent of the immunoreceptor tyrosine-based activation motifs (ITAMs), which are tyrosine phosphorylated during signalling by multichain immune recognition receptors (Nagel *et al*., 1993; Skubitz *et al*., 1995), while the CD66a sequence is instead reminiscent of an inhibitory ITIM (Beauchemin *et al*., 1997). Clustering of the ITAM-containing T cell receptor complex has, for example, been shown to activate T cells via Src family kinases (Kolanus *et al*., 1993), while co-activation of the ITIM receptor can inhibit this response (Olcese *et al*., 1996). It is interesting in this respect that adherence to the HeLa cell lines can result in different outcomes, as bacterial uptake after binding to HeLa-CD66d is up to 20-fold higher than that by the other cell lines (Fig. 1). CD66 proteins associate with Lyn and Hck of the Src kinase family (Skubitz *et al*., 1995), and CD66a is associated with the SHP-1 phosphatase (Beauchemin *et al*., 1997), suggesting that they are active in intracellular signalling events. Similarly, the Opa_{52}-expressing gonococci and *E. coli* strains used in this study trigger a signalling cascade, which involves the Src-like kinases, Hck and Fgr, and the small G-protein Rac1 (C. R. Hauck, T. F. Meyer, F. Lang and E. Gulbins, unpublished). This event can be mimicked by receptor cross-linking using anti-CD66 $F(ab)_2$ fragments, and inhibition of either Src family kinase or Rac1 function prevents bacterial uptake. Whether the differential binding of CD66a, CD66c and CD66d receptors by various Opa proteins can modulate the intracellular signalling and/or bactericidal response of phagocytes to bacterial adherence thus still remains an intriguing and important issue towards the further understanding of the gonococcal infection process.

Experimental procedures

Cell lines and bacterial strains

The stably transfected HeLa cell lines expressing defined recombinant CD66 proteins have been described previously (Berling *et al*., 1990; Nagel *et al*., 1993; Gray-Owen *et al*., 1997). HeLa cell lines were grown in RPMI-1640 with L-glutamine (Life Technologies) with 10% fetal calf serum (FCS) and 500 $\mu g\,ml^{-1}$ geneticin. The mutant cell line CHO-*pgs*677, which is defective in proteoglycan biosynthesis (Esko, 1991) was generously provided by Dr J. D. Esko (University of Alabama at Birmingham, USA). CHO cell lines were cultured in MEMα growth medium (Life Technologies) containing 10% FCS. The preparation of human umbilical vein endothelial cells (HUVECs) has been described previously (Dehio *et al*., 1997), and these were propagated in endothelial growth medium (Promocell). HUVECs were treated with 10 $ng\,ml^{-1}$ TNFα for 3–4 days where indicated. All cell lines were grown in a humidified atmosphere at 37°C with 5% CO_2. Transient transfection of CHO cells with the CD66a expression construct (Gray-Owen *et al*., 1997) was performed using lipofectamine reagent (Life Technologies) according to the company's protocol.

Recombinant strains invariantly synthesizing the 11 genetically defined Opa proteins of *N. gonorrhoeae* MS11 were described by Kupsch *et al*. (1993). The cloned *opa* genes were expressed in the genetic background of MS11 strain N279, which lacks pili and carries a deletion in the epithelial cell invasion-associated *opaC* locus. Gonococci were subcultured daily using a binocular microscope to monitor opacity phenotypes. The Hermes-10 expression vector was used for IPTG-inducible expression of gonococcal Opas in *E. coli* DH5 (Kupsch *et al*., 1996). Confirmation of Opa expression patterns was performed by immunoblot analysis of total bacterial extracts obtained from cultures used for infection and receptor isolation experiments using the Opa cross-reactive monoclonal antibody 4B12C11 (Achtman *et al*., 1988). Surface expression of the Opa proteins by recombinant *E. coli* strains has been confirmed by their proteolytic susceptibility in intact bacteria (Kupsch *et al*., 1993).

Infection of cells and microscopic and FACS analysis

All infection experiments were performed as outlined previously (Gray-Owen *et al*., 1997), except that infection with recombinant *E. coli* strains was allowed to continue for 5 h, and 100 μM IPTG was included in the cell culture medium. Immunocytochemistry, confocal laser scanning microscopy and phase contrast microscopy for the visualization of bacterial interactions with stably transfected HeLa or transiently transfected CHO cell lines was also performed as described previously (Gray-Owen *et al*., 1997). The anti-CD66 monoclonal antibody, D14HD11, which cross-reacts with CD66a, c, d and e, and CD66e-specific mAb 26/3/13 were generously provided by Dr Fritz Grunert (University of Freiburg, Freiburg, Germany). Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit and Texas red-conjugated goat anti-mouse antibodies were purchased from Dianova.

Isolation of Opa-specific receptor proteins from transfected HeLa cell lines

Isolation of Opa-specific receptor proteins using whole bacteria was accomplished as outlined previously (Gray-Owen *et al*., 1997), except that Triton X-100-soluble extracts were prepared instead from adherent HeLa cells expressing defined recombinant CD66 proteins. Recombinant *E. coli* strains expressing defined gonococcal Opas were diluted to an OD_{550} of 1 in phosphate-buffered saline (PBS) with 1 mM $MgCl_2$ and 0.5 mM $CaCl_2$ (PBS/Mg/Ca). An aliquot of 50 μl of cell extract was added to 450 μl of the bacterial suspension, and samples were incubated at 4°C for 2 h with gentle agitation. Bacteria were recovered by centrifugation and then washed for 3 × 10 min with PBS/Mg/Ca containing 0.2% Triton X-100 at 4°C. The washed bacterial pellet was resuspended in 1 pellet volume of PBS, diluted in 2 × SDS–PAGE sample buffer containing 1% 2-mercaptoethanol and boiled for 10 min

before electrophoresis. After immunoblotting, the samples were probed with either the Opa-specific monoclonal antibody 4B12C11 (Achtman *et al*., 1988; generously provided by Dr Mark Achtman, MPI-MG, Berlin, Germany) or the CD66 cross-reactive monoclonal antibody CLB/gran10 (Hiss Diagnostics) and then detected by horseradish peroxidase-conjugated goat anti-mouse IgG antibody.

Luminol-enhanced chemiluminescence assays

Human neutrophils were isolated from fresh citrated blood of healthy donors by gradient centrifugation on Ficoll Hypaque as described by Brandt *et al*. (1991). All preparations contained >95% neutrophils, as determined by analyses using an F800 cell counter, and cell viability was >99%, as determined by Trypan blue exclusion. Oxidative response was measured by luminol-enhanced chemiluminescence (CL) in a six-channel biolumat LB9505 (Berthold) at 37°C, essentially as described by Rest and Speert (1994). Neutrophils (4×10^5) were suspended in 100 μl of PBS supplemented with 5 mM glucose and 0.05% very low endotoxin BSA (Serva) and then preincubated at 37°C for 30 min before dilution with 400 μl of the same buffer supplemented with 0.5 mM $CaCl_2$, 1 mM $MgCl_2$ and 100 μM luminol. After 2 min of recording background CL levels, infection was begun by the addition of 8×10^6 bacteria freshly resuspended from overnight culture. Bacteria-induced CL was monitored continuously over a 60 min period. For each assay, uninfected cells were included as a negative control to determine spontaneous CL production, and cells stimulated with 1 μg ml^{-1} phorbol myristate acetate were used as a positive control. CL response was evaluated as the integral CL occurring between 10 and 60 min, and results are expressed as relative CL response compared with that induced by the Opa-negative control strains.

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References

Achtman, M., Neibert, M., Crowe, B.A., Strittmatter, W., Kusecek, B., Weyse, E., Walsh, M.J., Slawig, B., Morelli, G., Moll, A., and Blake, M. (1988) Purification and characterization of eight class 5 outer membrane protein variants from a clone of *Neisseria meningitidis* serogroup A. *J Exp Med* **168:** 507–525.

Beauchemin, N., Kunath, T., Robitaille, J., Chow, B., Turbide, C., Daniels, E., and Veillette, A. (1997) Association of biliary glycoprotein with protein tyrosine phosphatase SHP-1 in malignant colon epithelial cells. *Oncogene* **14:** 783–790.

Belland, R.J., Chen, T., Swanson, J., and Fischer, S.H. (1992) Human neutrophil response to recombinant neisserial Opa proteins. *Mol Microbiol* **6:** 1729–1737.

Berling, B., Kolbinger, F., Grunert, F., Thompson, J.A., Brombacher, F., Buchegger, F., von Kleist, S., and Zimmerman, W. (1990) Cloning of a carcinoembryonic antigen gene family member expressed in leukocyte of chronic myeloid leukemia patients and bone marrow. *Cancer Res* **50:** 6534–6539.

Bhat, K.S., Gibbs, C.P., Barrera, O., Morrison, S.G., Jahnig, F., Stern, A., Kupsch, E.-M., Meyer, T.F., and Swanson, J. (1991) The opacity proteins of *Neisseria gonorrhoeae* strain MS11 are encoded by a family of 11 complete genes. *Mol Microbiol* **5:** 1889–1901.

Bos, M.P., Grunert, F., and Belland, R.J. (1997) Differential recognition of members of the carcinoembryonic antigen family by Opa variants of *Neisseria gonorrhoeae*. *Infect Immun* **65:** 2353–2361.

Brandt, E., van Damme, J., and Flad, H.D. (1991) Neutrophils can generate their activator neutrophil-activating peptide 2 by proteolytic cleavage of platelet-derived connective tissue-activating peptide III. *Cytokine* **3:** 311–321.

Chen, T., and Gotschlich, E.C. (1996) CGM1a antigen of neutrophils, a receptor of gonococcal opacity proteins. *Proc Natl Acad Sci USA* **93:** 14851–14856.

Chen, T., Belland, R., Wilson, J., and Swanson, J. (1995) Adherence of $pilus^-$ Opa^+ gonococci to epithelial cells *in vitro* involves heparan sulfate. *J Exp Med* **182:** 511–517.

Chen, T., Grunert, F., Medina-Marino, A., and Gotschlich, E.C. (1997) Several carcinoembryonic antigens (CD66) serve as receptors for gonococcal opacity proteins. *J Exp Med* **185:** 1557–1564.

Dehio, C., Meyer, M., Berger, J., Schwarz, H., and Lanz, C. (1997) Interaction of *Bartonella henselae* with endothelial cells results in bacterial aggregation on the cell surface and the subsequent engulfment and internalisation of the bacterial aggregate by a unique structure, the invasome. *J Cell Sci* **110:** 2141–2154.

Densen, P., and Mandell, G.L. (1978) Gonococcal interactions with polymorphonuclear neutrophils. Importance of the phagosome for bactericidal activity. *J Clin Invest* **62:** 1161–1171.

Duensing, T.D., and van Putten, J.P. (1997) Vitronectin mediates internalisation of *Neisseria gonorrhoeae* by Chinese hamster ovary cells. *Infect Immun* **65:** 964–970.

Eades-Perner, A.-M., van der Putten, H., Hirth, A., Thompson, J., Neumaier, M., von Kleist, S., and Zimmerman, W. (1994) Mice transgenic for the human carcinoembryonic antigen gene maintain its spatiotemporal expression pattern. *Cancer Res* **54:** 4169–4176.

Esko, J.D. (1991) Genetic analysis of proteoglycan structure, function and metabolism. *Curr Opin Cell Biol* **3:** 805–816.

Farrel, C.F., and Rest, R.F. (1990) Up-regulation of human neutrophil receptors for Neisseria gonorrhoeae expressing PII outer membrane proteins. *Infect Immun* **58:** 2777–2784.

Fischer, S., and Rest, R.F. (1988) Gonococci possessing only certain P.II outer membrane proteins interact with human neutrophils. *Infect Immun* **56:** 1574–1579.

Gomez-Duarte, O.G., Dehio, M., Guzman, A.C., Chhatwal, G.S., Dehio, C., and Meyer, T.F. (1997) Binding of vitronectin to Opa-expressing *Neisseria gonorrhoeae* mediates invasion of HeLa cells. *Infect Immun* **65:** 3857–3866.

Gray-Owen, S.D., Dehio, C., Haude, A., Grunert, F., and

Meyer, T.F. (1997) CD66 carcinoembryonic antigens mediate interactions between Opa-expressing *Neisseria gonorrhoeae* and human polymorphonuclear phagocytes. *EMBO J* **16:** 3435–3445.
Handsfield, H.H. (1975) Disseminated gonococcal infection. *Clin Obstet Gynecol* **18:** 131–142.
Holmes, K.K., Counts, G.W., and Beaty, H.N. (1971) Disseminated gonococcal infection. *Ann Intern Med* **74:** 979–993.
Jantscheff, P., Nagel, G., Thompson, J., Kleist, S.V., Embleton, M.J., Price, M.R., and Grunert, F. (1996) A CD66a-specific, activation-dependent epitope detected by recombinant human single chain fragments (scFvs) on CHO transfectants and activated granulocytes. *J Leuk Biol* **59:** 891–901.
Jerse, A.E., and Rest, R.F. (1997) Adhesion and invasion by the pathogenic neisseria. *Trends Microbiol* **5:** 217–221.
King, G.J., and Swanson, J. (1978) Studies on gonococcus infection. XV. Identification of surface proteins of *Neisseria gonorrhoeae* correlated with leukocyte association. *Infect Immun* **21:** 575–583.
Klein, M.L., McGhee, S.A., Baranian, J., Stevens, L., and Hefta, S.A. (1996) Role of non-specific cross-reacting antigen, a CD66 cluster antigen, in activation of human granulocytes. *Infect Immun* **64:** 4574–4579.
Kolanus, W., Romeo, C., and Seed, B. (1993) T cell activation by clustered tyrosine kinases. *Cell* **74:** 171–170.
Kuijpers, T.W., Tool, A.T.J., van der Schoot, C.E., Gensel, L.A., Onderwater, J.J.M., Roos, D., and Verhoeuen, A.J. (1991) Membrane surface antigen expression on neutrophils: a reappraisal of the use of surface markers for neutrophil activation. *Blood* **78:** 1105–1100.
Kuijpers, T.W., Hoogerwerf, M., van der Laan, L.J.W., Nagel, G., van der Schoot, C.E., Grunert, F., and Roos, D. (1992) CD66 non-specific cross-reacting antigens are involved in neutrophil adherence to cytokine-activated endothelial cells. *J Cell Biol* **118:** 457–466.
Kupsch, E.-M., Knepper, B., Kuroki, T., Heuer, I., and Meyer, T.F. (1993) Variable opacity (Opa) outer membrane proteins account for the cell tropisms displayed by *Neisseria gonorrhoeae* for human leukocytes and epithelial cells. *EMBO J* **12:** 641–650.
Kupsch, E.-M., Aubel, D., Gibbs, C.P., Kahrs, A.F., Rudel, T., and Meyer, T.F. (1996) Construction of Hermes shuttle vectors: a versatile system useful for genetic complementation of transformable and non-transformable Neisseria mutants. *Mol Gen Genet* **250:** 558–569.
McGee, Z.A., Johnson, A.P., and Taylor-Robinson, D. (1981) Pathogenic mechanisms of *Neisseria gonorrhoeae*: observations on damage to human fallopian tubes in organ culture by gonococci of colony type 1 or 4. *J Infect Dis* **143:** 413–422.
McGee, Z.A., Stephens, D.S., Hoffman, L.H., Schlech, W.F., and Horn, R.G. (1983) Mechanisms of mucosal invasion by pathogenic Neisseria. *Rev Infect Dis* **5:** 708–714.
Majuri, M.-L., Hakkarainen, M., Paavonen, T., and Renkonen, R. (1994) Carcinoembryonic antigen is expressed on endothelial cells. *APMIS* **102:** 432–438.
Meyer, T.F., and van Putten, J.P.M. (1989) Genetic mechanisms and biological implications of phase variation in pathogenic Neisseriae. *Clin Microbiol Rev* **2:** S139-S145.
Nagel, G., Grunert, F., Kuijpers, T.W., Watt, S.M., Thompson, J., and Zimmerman, W. (1993) Genomic organization, splice variants and expression of CGM1, a CD66-related member of the carcinoembryonic antigen gene family. *Eur J Biochem* **214:** 27–35.
Naids, F.L., and Rest, R.F. (1991) Stimulation of human neutrophil oxidative metabolism by non-opsonized *Neisseria gonorrhoeae*. *Infect Immun* **59:** 4383–4390.
Naumann, M., Wessler, S., Bartsch, C., Wieland, B., and Meyer, T.F. (1997) *Neisseria gonorrhoeae* epithelial cell interaction leads to the activation of the transcription factors NF-kB and AP1 and the induction of inflammatory cytokines. *J Exp Med* **186:** 247–258.
Oikawa, S., Inuzuka, C., Kuroki, M., Arakawa, F., Matsuoka, Y., Kosaki, G., and Nakazato, H. (1991) A specific heterotypic cell adhesion activity between members of carcinoembryonic antigen family, W272 and NCA, is mediated by N-domains. *J Biol Chem* **266:** 7995–8001.
Olcese, L., Lang, P., Vely, F., Cambiaggi, A., Marguet, D., Blery, M., Hippen, K.L., Biassoni, R., Moretta, A., Cambier, J.C., and Vivier, E. (1996) Human and mouse killer-cell inhibitory receptors recruit PTP1C and PTP1D protein tyrosine phosphatases. *J Immunol* **156:** 4531–4534.
Prall, F., Nollau, P., Neumaier, M., Haubeck, H.-D., Drzeniek, Z., Helmchen, U., Loning, T., and Wagener, C. (1996) CD66a (BGP), an adhesion molecule of the carcinoembryonic antigen family, is expressed in epithelium, endothelium, and myeloid cells in a wide range of normal human tissues. *J Histochem Cytochem* **44:** 35–41.
Rest, R.F., and Speert, D.P. (1994) Measurement of non-opsonic phagocytic killing by human and mouse phagocytes. *Methods Enzymol* **236:** 91–108.
Rudel, T., van Putten, J.P.M., Gibbs, C.P., Haas, R., and Meyer, T.F. (1992) Interaction of two variable proteins (PilE and PilC) required for pilus-mediated adherence of *Neisseria gonorrhoeae* to human epithelial cells. *Mol Microbiol* **6:** 3439–3450.
Skubitz, K.M., Campbell, K.D., Ahmed, K., and Skubitz, A.P.N. (1995) CD66 family members are associated with tyrosine kinase activity in human neutrophils. *J Immunol* **155:** 5382–5390.
Stocks, S.C., Ruchaud-Sparagano, M.-H., Kerr, M.A., Grunert, F., Haslett, C., and Dransfield, I. (1996) CD66: role in the regulation of neutrophil effector function. *Eur J Immunol* **26:** 2924–2932.
Stromberg, N., Deal, C., Nyberg, G., Normark, S., So, M., and Karlsson, K.-A. (1988) Identification of carbohydrate structures that are possible receptors for *Neisseria gonorrhoeae*. *Proc Natl Acad Sci USA* **85:** 4902–4906.
Sweet, R.L., Draper, D.L., and Hadley, W.K. (1981) Etiology of acute salpingitis: influence of episode number and duration of symptoms. *Obstet Gynecol* **58:** 62–68.
Thompson, J.A., Grunert, F., and Zimmerman, W. (1991) Carcinoembryonic antigen gene family: molecular biology and clinical perspectives. *J Clin Lab Anal* **5:** 344–366.
van Putten, J.P.M., and Paul, S.M. (1995) Binding of syndecan-like cell surface proteoglycan receptors is required for *Neisseria gonorrhoeae* entry into human mucosal cells. *EMBO J* **14:** 2144–2154.
Virji, M., and Heckels, J.E. (1986) The effect of protein II and pili on the interaction of *Neisseria gonorrhoeae* with human

polymorphonuclear leukocytes. *J Gen Microbiol* **132:** 503–512.

Virji, M., Makepeace, K., Ferguson, D.J.P., and Watt, S. (1996a) Carcinoembryonic antigens (CD66) on epithelial cells and neutrophils are receptors for Opa proteins of pathogenic neisseriae. *Mol Microbiol* **22:** 941–950.

Virji, M., Watt, S.M., Barker, S., Makepeace, K., and Doyonnas, R. (1996b) The N-domain of the human CD66a adhesion molecule is a target for Opa proteins of *Neisseria meningitidis* and Neisseria gonorrhoeae. *Mol Microbiol* **22:** 929–939.

Ward, M., Watt, P.J., and Robertson, J.N. (1974) The human fallopian tube: a laboratory model for gonococcal infection. *J Infect Dis* **129:** 650–659.